HORMONAL REGULATION AND METABOLIC ROLES OF CCAAT/ENHANCER-BINDING PROTEINS

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ABSTRACT

The CCAAT/Enhancer-Binding proteins (C/EBPs) are liver-enriched transcription factors which are known to *trans*-activate a number of metabolically important genes. The goal of this thesis work has been to advance areas of study on C/EBP isoform regulation and metabolic roles which have not been fully addressed in the current literature.

The initial undertaking of this work involved the examination of the effects of hormones and diabetes on C/EBP isoform expression in rat H4IIE hepatoma cells and in rat liver. Treatment of cells with dexamethasone was observed to produce increases in C/EBPα and C/EBPβ mRNA and protein levels. Insulin was observed to produce an interesting bi-phasic response on C/EBPα expression. Treatment of H4IIE cells with 8-chlorophenylthio-cAMP produced greater inductive effects upon C/EBPβ expression than on C/EBPα expression. We observed an inhibition of C/EBPα gene expression in streptozotocin-diabetic rat liver which was reflected by decreases in both its mRNA and protein. However, an interesting alteration in the ratio of alternate C/EBPα translation forms was observed in the streptozotocin-diabetic livers suggesting a potential alteration in the *trans*-activational activity of C/EBPα. These results suggest that hepatic C/EBP isoforms are under complex control by both hormonal and metabolic signals, which correlates well with their known role as *trans*-activators of metabolically vital genes.

Previous work has demonstrated a role for C/EBPα in mediating the cAMP responsiveness of synthetic phosphoenolpyruvate carboxykinase (PEPCK) promoter constructs within a transiently transfected cell culture system. In order to address the

C/EBP isoform requirements for endogenous PEPCK gene expression and regulation, we have produced stable transfected hepatoma cells expressing antisense constructs for the two major C/EBP isoforms in liver. We demonstrate that targeted inhibition of C/EBPα but not C/EBPβ in rat hepatoma H4IIE cells significantly reduces the cAMP responsiveness of the endogenous PEPCK promoter. Cells expressing C/EBPα antisense were characterized by decreases in the levels C/EBPα mRNA and C/EBPα protein levels. The response of PEPCK to cAMP was marginal in C/EBPα antisense expressing cells, compared with a 3-fold induction of PEPCK expression by cAMP observed in wild-type H4IIE cells. The cAMP signaling pathway of C/EBPα antisense expressing cells was intact in that the cAMP induction of the C/EBPβ gene was similar to that of normal H4IIE cells. Furthermore, the cAMP responsiveness of PEPCK in C/EBPβ antisense expressing cells was nearly identical to that of wild-type H4IIE cells. These data suggest that the α-isoform of C/EBP is specifically required for mediation of the cAMP response of endogenous PEPCK in rat hepatoma cells and cannot be functionally substituted for by C/EBPβ in this context.

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My thanks are also extended to my "A13 lab bud" and friend Jerry Davies who I am greatly indebted to not only for helping to produce a rather interesting laboratory experience, but also to have provided myself with the opportunity to avail from his considerable technical experience and friendship. I must point out that Mr. Davies is responsible for producing the photographed results presented in figures 4.4 and 4.7 of this thesis, both of which were quite pivotal to this work.

I must also acknowledge the two ladies of the Roesler laboratory, Pamela McFie and Heather Laidlaw, who on both professional and personal levels and by their own natures, have made my time in their company seem just that much brighter.

My greatest thanks also goes out to the many friends whom I have encountered throughout my life, who near or far, dear or not so dear, have one way or the other contributed to the experiences which have made me who I am and brought me to this stage of the game. Despite the time or distance which may ultimately separate us, I will see you all again someday.

Finally, I must now attempt to extend the kind of love and thanks to which words can never do justice. The two most indispensable people in my life, my parents, have quite simply never let me down. Their strong encouragement and sound advise have always and will always serve me well in life. My hope is that I can repay their love and belief with the same in kind, and to someway instill in them a sense of pride in their son which will never falter.

This work is dedicated to the memory of my grandfather

Wilfred Oakley Crosson

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LIST OF ABBREVIATIONS

8-CPT-cAMP	8 Chlorophenylthio-Cyclic Adenosine Monophosphate
αA B1	C/EBPα Antisense RNA Expressing Stable Transfected H4IIE Clone B1
AE	Accessory Element
AF	Accessory Factor
AGP	α_1-acid Glycoprotein Gene
ATF-2	Activating Transcription Factor - 2
βA C4	C/EBPβ Antisense RNA Expressing Stable Transfected H4IIE Clone C4
bp	Base pair
BSA	Bovine Serum Albumin
bZIP	Basic Region - Leucine Zipper
C/EBP	CCAAT/Enhancer-Binding Protein
cAMP	3'-5'-Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CHOP-10	C/EBP Homologous Protein
ChoRE	Carbohydrate Response Element
Ci	Curie
COUP-TF	Chicken Ovalbumin Upstream Promoter Transcription Factor
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
CRU	cAMP Response Unit
dATP	2'-Deoxyadenosine 5'-Triphosphate
DBP	D-Site Binding Protein

dCTP	2'-Deoxycytosine 5'-Triphosphate
ddATP	2' 3'-Dideoxyadenosine 5'-Triphosphate
ddCTP	2' 3'-Dideoxycytosine 5'-Triphosphate
ddGTP	2' 3'-Dideoxyguanosine 5'-Triphosphate
ddTTP	2' 3'-Dideoxythymidine 5'-Triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-Deoxyguanosine 5'-Triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DTE	Dithioerythritol
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5'-Triphosphate
EDTA	Ethylene-diamine Tetraacetic Acid Disodium Salt
G418	Gentamicin Sulphate
GADD 153	Growth Arrest and DNA Damage Inducible Gene (C/EBPζ)
GαN175 A3	C/EBPα Sense RNA Expressing Stable Transfected H4IIE Clone A3
GBF-1	G-box Binding Factor - 1
GBF-F D4	GBF-F Expressing Stable Transfected H4IIE Clone D4
GDP	Guanosine Diphosphate
GLUT-4	Insulin-sensitive Glucose Transporter
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GRU	Glucocorticoid Response Unit
GTFs	General Transcription Factors
GTP	Guanosine Triphosphate

H4-wt	Wild Type Rat Hepatoma H4IIE Cells
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid
HNF-4	Hepatic Nuclear Factor - 4
HRE	Hormone Response Element
HRU	Hormone Response Unit
IL	Interleukin
IRF	Insulin Response Factor
IRS	Insulin Response Sequence
kb	Kilobase Pair
kDa	Kilodalton
LAP	Liver Activator Protein
LB	Luria-Bertani Medium
LIP	Liver Inhibitory Protein
LPS	Lipopolysaccharide
LSR	Liver Specific Region
MOPS	(3-[N-Morpholino]propanesulfonic Acid)
mRNA	Messenger RNA
MSV	Moloney Murine Sarcoma Virus
NF-1	Nuclear Factor - 1
NF-IL6	Nuclear Factor for Interleukin-6 Expression (C/EBPβ)
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG 8000	Polyethylene Glycol 8000
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate Carboxykinase

PIPES	1,4-Piperazine Diethane Sulfonic Acid, 1.5 Sodium Salt
PKA	Protein Kinase A
PKB/Akt	Protein Kinase B
PMSF	Phenylmethylsulfonyl Flouride
PPAR	Peroxisome Proliferator Activated Receptor
PPARRE	Peroxisome Proliferator Activated Receptor Response Element
PVP	Polyvinylpyrrolidone
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Receptor Response Element
RIPA	Radio-Immuno-Precipitation Assay
RNA pol II	RNA Polymerase II
RNA	Ribonucleic Acid
rNTPs	Ribonucleotide Triphosphates
rRNA	Ribosomal RNA
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulphate
TAFs	TATA Binding Protein Associated Factors
TBP	TATA Binding Protein
TEMED	N,N,N',N'-Tetramethylenediamine
TNFα	Tumor Necrosis Factor α
TR	Thyroid Hormone Receptor
TRE	Thyroid Hormone Response Element
Tris	Tris-[hydroxymethyl]aminomethane
UTP	Uridine Triphosphate

1. INTRODUCTION

The regulation of gene expression in higher organisms is a complex biological process. Multi-cellular organisms are capable of both intra- and extra-cellular communication which can serve as the cue for modulation of gene expression. The ability of higher eukaryotes to modulate their patterns of gene expression in cell-specific fashion in response to physiological signals sets them apart from single cellular organisms in the complexity of their behavior, and of course, the complexity of their regulatory mechanisms.

This work deals with several nuclear transcription factors known as the CCAAT/Enhancer-Binding proteins (C/EBPs), more specifically, the α and β-isoforms which are highly expressed in liver. Given that C/EBP isoforms play an important role in the hormonal regulation of a number of metabolically relevant genes, it stands to reason that they might also be hormonally regulated. The initial purpose of this thesis was to define the effects of a number of hormones upon C/EBP isoform expression both in cell culture, and to what extent was possible, *in vivo*. During the course of this initial work it became apparent that C/EBPα played a vital role in mediating the cAMP responsiveness of phosphoenolpyruvate carboxykinase (PEPCK) gene promoter constructs in human hepatoma cells (Roesler, *et al.*, 1996). The objectives of this research project then expanded to address the role of C/EBP isoforms in the cAMP responsiveness of PEPCK

gene expression in the intact hepatoma cell, utilizing both antisense and dominant negative inhibitor methodologies to this end. As the results of this research will show, C/EBP isoform expression is regulated by a number of metabolically relevant hormones in liver, and C/EBPα is vital for the mediation of cAMP responsiveness of the PEPCK promoter.

In order to appropriately consider the relevant literature prior to demonstrating the results of this work, section 2 of this document will first consider the basic concepts of transcriptional regulation of gene expression (section 2.1). Section 2.2 will deal with hormonal regulation over a gene's promoter in greater depth, using the PEPCK promoter as a relevant example. Section 2.3 will deal with C/EBP in detail, in terms of its biochemistry, tissue specific and hormonally regulated expression, and its physiological roles. The following section (2.4) will deal with the background knowledge concerning the utilization of antisense constructs and dominant negative inhibitors to modulate transcription factor function. Finally, section 2.5 will describe the specific objectives of this research project.

2. REVIEW OF THE LITERATURE

2.1 Regulation of Gene Expression

2.1.1 Overview of Transcriptional Regulation (*cis* and *trans*)

Ultimately, it is the initiation of messenger RNA (mRNA) synthesis by RNA polymerase II (RNA pol II) in mammalian systems which is a main control point for the regulation of gene expression. From a basic viewpoint, adaptive changes in expression of a given gene induced by relevant physiological stimuli, result from the interaction of an enormous group of nuclear proteins known as transcription factors, or *trans*-acting factors, with specific regions or *cis*-elements, of a gene's promoter. Spatially, eukaryotic *cis*-elements for RNA pol II transcribed genes can be located either upstream or downstream of the transcriptional initiation site. The location, number and arrangement of the *cis*-elements varies from gene to gene. It is the character and arrangement of the *cis*-elements which can define the possible forms of regulation over a given gene. DNA *cis*-elements serve as the binding or nucleation sites for groups of transcription factors which *via* protein – protein interactions, potentially with the RNA pol II complex itself, effect either positively, or negatively, the rate of transcriptional initiation.

The *cis*-elements of the promoter region of most sequenced genes occur within the first 1000 base pairs (bp) of the transcriptional start site. *Cis*-elements located at greater distances (greater than 1000 bp, and up to perhaps even 30 kilobase pairs (kb)), and which

can carry out regulation in an orientation – independent manner are by convention known as enhancer elements. Refer to Figure 2.1 for a diagram of a basic promoter.

Potentially, *trans*-factors binding to remote enhancer elements might still interact with other factors binding to the main promoter, or with the RNA pol II complex directly by DNA bending. Certain *cis*-elements are common to many pol II transcribed genes, for example GC and CCAAT boxes and the TATA element. Other *cis*-elements will be present based on the nature of the regulatory properties of the gene. Often, certain *cis*-elements correspond to a "consensus sequence" for binding of transcription factors involved in specific forms of signal-transduced regulation. However, although factors which participate in certain forms of stimuli may bind a given consensus sequence, they are not limited to binding this sequence alone. In other words, the concept of consensus sequence might be incorrect in that it is not the case that only certain *trans*-factors bind certain *cis*-elements. The actual situation has proven to be far more complex, since numerous examples have shown that transcription factor association with a given *cis*-element is often mediated by other factors *via* protein-protein interactions. In fact, multiple factors may bind the same DNA sequence, producing a competitive phenomenon, and some *trans*-factors may not bind DNA at all but associate only with other directly bound, or otherwise interacting factors. It also appears to be the case that some signals, particularly those generated by certain hormones, are often mediated through a number of factors binding to multiple promoter elements (for reviews see Lucas and Granner, 1992 and Roesler and Park, 1998).

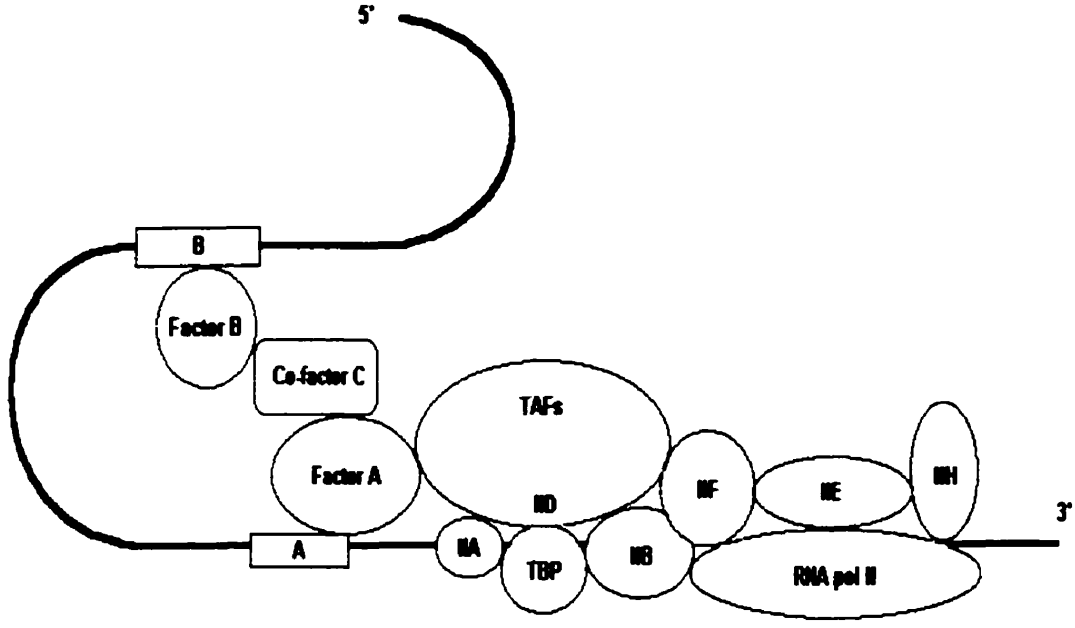

Figure 2.1 Basic schematic diagram of a hypothetical TATA-containing promoter showing general transcription factors (including TFIID, composed of the TATA-binding protein (TBP) and associated TAFs, and other general transcription factors including TFIIA, TFIIB, TFIIF, TFIIE and TFIIH) clustered around and/or in contact with RNA polymerase II (RNA pol II), forming the pre-initiation complex. DNA binding factor A is affecting transcription through direct interaction with the pre-initiation complex. Transcription is further affected by the influence of DNA bound factor B, and the co-factor C which is physically linking the two DNA-bound factors and the pre-initiation complex with the aid of DNA looping.

Transcription factors are for the most part, but not always, sequence specific DNA binding proteins. These factors are expressed in low abundance in the nucleus, although certain factors are known to be localized to the nucleus only upon being exposed to an appropriate physiological signal (considered by Vandromme, *et al.* 1996), for example C/EBPβ is known to translocate from the cytoplasm to the nucleus upon an increase in the intracellular levels of cAMP in rat PC12 cells (Metz and Ziff, 1991). In general, transcription factors can be thought of as being modular in nature, composed of separable DNA binding and transcriptional activation domains.

DNA binding domains are quite compact, usually consisting of less than 100 amino acids. The four most commonly identified DNA binding motifs are the leucine zipper – basic region (bZIP) motif, the zinc finger, the helix – loop – helix motif, and the homeodomain motif (Mitchell and Tjian, 1989). As the bZIP motif was first characterized from analysis of the DNA binding domain of the CCAAT/Enhancer-Binding protein (Johnson, *et al.* 1987), in which this thesis is primarily concerned, we shall consider it in some detail at this point. Quite simply, the basic region – leucine zipper consists of a bipartite region of sequence; a stretch of approximately 30 amino acids which has an overall net basic charge, followed by a amino acid sequence which possesses a heptad repeat of leucine residues. This " leucine zipper" is required for dimerization among certain bZIP family members, as well as for DNA binding (Landschultz, *et al.* 1988b). The dimerization between two monomers is thought to be stabilized by hydrophobic interactions between opposing α-helical regions of the leucine repeats in a matter analogous to the formation of quaternary coiled-coils (Crick, 1953) by such proteins as

keratin and myosin heavy chain. Cross dimerization can occur between divergent members of the bZIP family, however the nature of the interactions are based on leucine zipper homology and the effects of covalent modification. For example, C/EBPα and C/EBPβ (LAP) share considerable sequence homology in their DNA binding and dimerization domains and are known to form heterodimers *in vitro* (Williams, *et al.* 1991), and may also form covalently linked dimers by disulfide bonding concomitant with the formation of the coiled-coil. The ability of bZIP family members to cross-dimerize allows for an exponential increase in the combinations of possible DNA binding specificities, as well as *trans*-activational properties, from a limited set of nuclear proteins. Section 2.3.1.1 of this thesis will deal with the basic region - leucine zipper domain of C/EBP in greater detail.

Much like DNA-binding domains, activation domains are normally relatively short segments of primary sequence, usually less than 100 amino acids in length. Many factors are known to have more than one activation domain, and these separate domains may often act in synergy with one another, whereas, an isolated activation domain may have little, if any, *trans*-activational capability. The three main activation domains which have been identified in the majority of *trans*-acting factors are the acidic - α-helical, glutamine-rich, and proline-rich motifs (Ptashne, 1988). The general consensus is that these regions exhibit their functionality by making contacts with other proteins, presumably with members of the general pre-initiation complex (the general transcription factors or GTFs), and then in some manner stabilize interactions with DNA or with other proteins, and thereby favor the initiation of transcription. The *trans*-activation domains of C/EBP will be discussed in further detail in section 2.3.1.2.

2.1.2 Hormone Response Units

The overall organization of *cis*-elements, with which *trans*-acting factors relevant to the mediation of a given hormonal signal associate with, have come to be known as hormone response units (HRUs) (Lucas and Granner, 1992). However, this statement in itself is somewhat too simplistic, as many metabolically important gene products are known to be regulated *via* integration of a number of different physiological stimuli. Nevertheless, because of the complex nature of HRUs, they can allow both negative cooperativity, and synergy between different signaling pathways (Roesler and Park, 1998). This convergence of multiple signaling pathways allows for a higher order of regulation. Hormonal response units typically consist of one or more recognizable hormone response elements (HREs) as well as a number of accessory elements (AE's). In general, a HRE in isolation is able to mediate a recognizable transcriptional effect in response to a relevant hormonal signal. However, this is usually not the case for AE's in isolation; rather AE's would seem to confer amplification or even permissivity to the transcriptional response. As reviewed by Lucas and Granner (1992), HREs and AEs taken together as a HRU allow for mechanisms to modulate the specificity, magnitude, and positive or negative polarity of a transcriptional response. In the next section (2.2) we will discuss in detail an example of a complex hormonally regulated gene promoter, the PEPCK promoter, upon which a significant portion of the work of this thesis resides.

2.2 The PEPCK Gene Promoter, a Paradigm of Hormonally Regulated Gene Expression

PEPCK is generally considered to be the rate-limiting enzyme of gluconeogenesis (Rognstad, 1979). The PEPCK enzyme catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and CO_2, requiring guanosine triphosphate as a cofactor:

$$\text{Oxaloacetate} + \text{GTP} \longleftrightarrow \text{PEP} + \text{GDP} + CO_2$$

Two forms of PEPCK enzyme exist. The mitochondrial form (PEPCK-M) is constitutively expressed and is the form predominantly active when lactate is used as a gluconeogenic precursor (Hanson and Patel, 1994). The cytosolic form (PEPCK-C) is under hormonal regulation, and is rather unique amongst the majority of regulatory enzymes in that it has no known allosteric modifiers. For this reason, control of gene transcription plays the predominate role in regulating PEPCK-C's level of cellular activity. It should be noted that two separate genes encode PEPCK-C and PEPCK-M, each with its own unique promoter region. The work described in this thesis deals only with the effects on the promoter of PEPCK-C.

2.2.1 Tissue-Specific Expression of PEPCK-C

The cytosolic form of PEPCK is expressed mainly in the liver and kidney, the two primary gluconeogenic tissues. However, expression is also detected in non-gluconeogenic

tissues such as small intestine, lung, mammary gland, and white and brown adipose (Ballard, *et al.* 1967; Anderson, 1970; Feldman and Hirst, 1978; Lobato, *et al.* 1985 and Zimmer and Magnuson, 1990). Presumably, PEPCK can be involved in glycerol sythesis during starvation *via* a process known as glyceroneogenesis as first described by Ballard, *et al.* (1967). Even during the net lipolysis which occurs during starvation, a certain level of triglyceride synthesis is required and PEPCK may act to ultimately produce 3-phosphoglycerate which can be utilized in the re-esterification of free fatty acids. PEPCK-C expression in kidney has a dual role due to the symbiotic relationship between gluconeogenesis and ammoniagenesis in this tissue during starvation (Alleyne, *et al.* 1969 and Pitts, 1974). A metabolic acidosis often results during starvation and this pH imbalance can be offset by ammonia production *via* the interconversion of glutamine to α-ketoglutarate. The α-ketoglutarate produced can be converted to oxaloacetate which is utilized by PEPCK to ultimately produce glucose, which is also required in the starvation state.

2.2.2 Physiological Factors Which Effect PEPCK-C Gene Expression in Liver

PEPCK expression can be modified by a number of influences, including diet, oxidation-reduction state (Hellkamp, *et al.* 1991 and Kietzmann, *et al.* 1992), physiological states such as starvation or diabetes, and the direct effects of various hormones including glucagon (*via* cAMP), glucocorticoids, thyroid hormone, and insulin. Correspondingly, PEPCK transcription in liver is up-regulated when physiological stimuli indicate that

increased glucose production is required, such as during starvation (Kioussis, *et al.* 1978), by metabolic aberration during diabetes (Sasaki, *et al.* 1984 and Friedman, *et al.* 1993), or in response to increasing levels of glucagon (Tilghman, *et al.* 1974) or glucocorticoids (Alleyne, *et al.* 1969). PEPCK transcription is strongly down-regulated by insulin (Tilghman, *et al.* 1974), prolonged high-carbohydrate diet (Lamers, *et al.* 1982), glucose (Kahn, *et al.* 1989 and Meyer, *et al.* 1991), and by a number of other compounds such as serotonin (Zabala, *et al.* 1992) and phorbol esters (O'Brien, *et al.* 1991).

2.2.3 Transcriptional Regulation of the PEPCK-C Gene

It should be understood that aside from the majority of regulation of PEPCK-C expression which occurs at the level of its transcription rate there is also some degree of control over the stability of its mRNA. PEPCK-C mRNA is rapidly degraded with a half-life of approximately 30 minutes (Nelson, *et al.* 1980). The mRNA of PEPCK can be stabilized by binding of a hepatocyte enriched protein to its 3' un-translated region. The levels of this protein are induced in liver in response to fasting (Nachaliel, *et al.* 1993). The half-life of PEPCK-C mRNA is also known to be extended to nearly 4 hours by the influence of a cAMP analogue (Hod and Hanson, 1988). These factors taken together suggest a mechanism for sustaining PEPCK mRNA levels and enzyme activity during the initial stages of starvation, and or in response to increased levels of cAMP.

2.2.3.1 The Hormonal Response Units of the Rat PEPCK-C Promoter

The majority of hormonal response units present in the rat PEPCK-C gene promoter can be found within the first 500 bp upstream of the transcriptional start site. To date, eleven distinct *cis*-elements have been identified within this region and are known to bind a multitude of liver expressed factors (See Figure 2.2 and Table 2.1 for summary). Outlying regions at -987 and -660 bp of the rat PEPCK-C promoter has been elucidated to be a peroxisome-proliferator-activated-receptor response elements (PPARREs), and are known to bind heterodimers of the peroxisome-proliferator-activated-receptor isoform γ_2 (PPARγ_2) and the retinoid-X receptor α-isoform (Tontonoz, *et al.* 1995). Overexpression of these factors, even in a non-adipose background, in the presence of promoter constructs containing the PPARE can lead to significant activation of PEPCK gene expression.

In the following sections we will consider the individual hormonal response units of the PEPCK-C promoter.

2.2.3.1.1 The Glucocorticoid Response Unit

Interestingly, while the PEPCK-C promoter is activated by glucocorticoids in liver (Meisner, *et al.* 1985) and inhibited in adipose tissue (Nechushtan, *et al.* 1987), there are no "consensus" glucocorticoid response elements present within the promoter. There are however, two sites which bind the glucocorticoid receptor (GR), known as GRE-1 and GRE-2 (Refer to Table 2.1). These dual response elements bind the glucocorticoid receptor weakly and are not sufficient to mediate the full transcriptional response of the

Table 2.1. Regulatory *cis*-elements of the PEPCK-C promoter

Binding Site	Location	Sequence	Binding Protein(s)
TATA	-29 / -23	TATTTAAA	TBP
CRE-1	-91 / -84	TTACGTCA	CREB, fos/jun, ATF-2, C/EBP
P1	-116 / -104	TGGCTATGATCCA	NF-1, CTF
CRE-2	-144 / -137	TTAGGTCA	C/EBP
P2	-190 / -185	ATTAAC	HNF-1
P3(I) - C site	-243 / -235	TTGTGTAAG	C/EBP
P3(II) - A site	-258 / -252	TTAGTCA	fos/jun
P4	-282 / -274	AATCAGCAAC	C/EBP
TRE	-332 / -316	TGGGGGTCAAGGACAGG	TR
GRE-2	-363 / -353	CATATGAAGTC	GR
GRE-1 (P5)	-385 / -374	ACACAAAATGTG	GR
AF-2 / IRS	-414 / -400	TGGTGTTTTGACAAC	HNF-3, C/EBP
AF-1 (P6)	-450 / -436	ATGACCTTTGGCCGT	HNF-4, COUP-TF, RARα
PPARRE	-450 / -436 -999 / -987	GATCCATGACCTTTGGCC GTGGGAG	$PPARg_2$ / RXR

This table is adapted from Liu and Hanson, 1991.

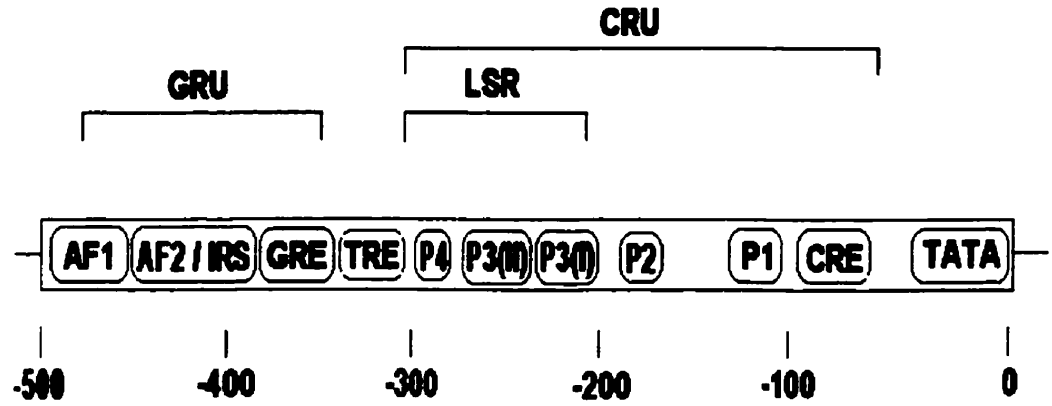

Figure 2.2 Spatial arrangement of the *cis*-elements of the rat PEPCK-C gene promoter. The following abbreviations are used: AF1, accessory factor 1 binding site; AF2/IRS, accessory factor 2 binding site / insulin response sequence; GRE, glucocorticoid response element; TRE, thyroid hormone response element; CRE, cAMP response element; GRU, glucocorticoid response unit; LSR, liver-specific region; CRU, cAMP response unit. This figure is adapted from Nizielski, *et al.* (1996).

PEPCK-C promoter to glucocorticoids. They require the involvement of two accessory factor sites, AF-1 and AF-2 (Insulin Responsive Sequence (IRS)), thus forming a complete glucocorticoid response unit (GRU) (Imai, *et al.* 1990). The AF-1 binding site is palindromic and consists of two half-sites, termed box b and c, which can serve as binding sites for hepatic nuclear factor - 4 (HNF-4) and the chicken ovalbumin upstream promoter - transcription factor (COUP-TF) (Hall, *et al.* 1995), one of which may be functionally redundant. The AF-1 site is also a retinoic acid response element (RARE), which can bind heterodimers of retenoic acid receptor α (RARα) and retenoid X receptor α (RXRα) (Hall, *et al.* 1992). However, the glucocorticoid response of the PEPCK-C promoter does not seem to be effected directly by RARα. The AF-2 (IRS) site seems to play a dual role, being involved in mediating glucocorticoid responsiveness, and the negative effects of insulin as well (Imai, *et al.* 1990 and O'Brien, *et al.* 1990). The IRS appears to be flanked on either side by binding sites for C/EBP and HNF-3 (O'Brien, *et al.* 1995 and O'Brien *et al.* 1994 respectively); either of these factors may be involved in glucocorticoid induction or insulin repression, and in the case of C/EBP, may be involved in linking these responses to other signaling pathways, such as the synergistic induction of PEPCK with cAMP. Discussion of the role of the IRS in the negative regulation of PEPCK-C expression will be discussed further in section 2.2.3.1.4.

2.2.3.1.2 The Thyroid Hormone Response Unit

Only two *cis*-elements of the PEPCK-C promoter are required for full responsiveness to thyroid hormone. The thyroid hormone receptor (TR) binds to the PEPCK thyroid response element (TRE) (Giralt, *et al.* 1991) as a heterodimer with the retinoid X receptor (RXR) (Mangelsdorf, *et al.* 1990 and 1995, and Schmidt, *et al.* 1993). The second required element is the P3(I) element to which either the α or β-isoforms of C/EBP can bind and participate in the thyroid response of PEPCK-C (Park, *et al.* 1999). Again, as occurs in the glucocorticoid response unit, the presence of a C/EBP isoform allows for synergism with the other response units of the promoter with which it interacts, potentiating the inductive effect upon PEPCK-C expression.

2.2.3.1.3 The cAMP Response Unit

The cAMP response unit of the PEPCK-C promoter consists of two independently weak elements, a cAMP response element (CRE-1), and an upstream liver-specific region (LSR) which contains binding sites for three C/EBP molecules and one molecule of the fos/jun heterodimer, AP1 (Roesler, *et al.* 1994). The cAMP response element (CRE-1) of the PEPCK-C promoter was first described by Bokar, *et al.* (1988), and as reviewed by Roesler, *et al.* (1988), was observed to be quite similar in sequence to CREs of other cAMP responsive genes. It should be noted that a second putative CRE, CRE-2, is located 45 base pairs upstream of CRE-1 (Roesler, *et al.* 1989). However, CRE-2 is not thought to participate directly in the cAMP responsiveness of PEPCK-C in rat liver. Multiple bZIP

transcription factor family members including the cAMP response element binding protein (CREB), C/EBP isoforms, D-site binding protein (DBP), jun/jun homodimers, fos/jun (AP1) heterodimers, and activating transcription factor-2 (ATF-2) are known to bind the CRE-1 and induce PEPCK-C transcription *in cyto* (Quinn, *et al.* 1990; Park, *et al.* 1993, Roesler, *et al.* 1992 and Cheong, *et al.* 1998). Initial affinity chromatography studies utilized to purify factors which could bind the CRE-1, revealed a 43-kDa protein which could correspond either to CREB or the α-isoform of C/EBP (Roesler, *et al.* 1989). Further DNA-protein binding assays demonstrated that the affinity of binding to the CRE-1 was much higher for C/EBPβ and CREB than was for C/EBPα (Park, *et al.* 1993). However, a recent study by Roesler, *et al.* (1998), has shown that the cAMP response of the PEPCK-C promoter can occur in the absence of CREB, and can be subsituted for by the α-isoform of C/EBP. Thus, there remains some controversy over the identity of the factor which binds CRE-1 and mediates cAMP responsiveness.

The upstream liver-specific region (LSR) of the PEPCK-C promoter was first described in detail by Roesler, *et al.* (1994) and confirmed by Yeagley, *et al.* (1998). Much like the CRE-1 in isolation, the LSR *cis*-elements are only capable of modulating a minimal response to cAMP by themselves; they rather serve to mediate the robust cAMP responsiveness of the PEPCK-C promoter by synergizing with the downstream CRE-1. The LSR was so named because the number of *trans*-acting factors which can bind to its sequences are enriched in liver nuclear extracts, such as C/EBP isoforms and the D-site binding protein. The LSR contains the originally described P(3) and P(4) *cis*-elements, the aforementioned work by Roesler, *et al.* (1994), has shown that the P(3) site is split into two

half sites, known as the A and C sites or as P3(II) and P3(I), respectively (refer to Table 2.1). The C site binds C/EBP isoforms and along with the two other C/EBP proteins binding to the P(4) region, are required for synergistic mediation of cAMP responsiveness along with CRE-1. The A site (P3(II)) serves to bind the fos/jun heterodimer AP1, and appears to act to augment the synergism between the complete LSR and CRE-1, since the A site alone has essentially no ability to synergize with the CRE-1. It should be noted that the necessity of an AP-1 binding site has also been demonstrated within the CRUs of a number of other gene promoters including c-*fos* (Fisch, *et al.* 1989) and the proenkephalin gene (Hyman, *et al.* 1989).

A final note in this brief primer on the cAMP response unit of the PEPCK-C promoter, is the potential existence of a "braking mechanism" over protein kinase A (PKA) inducible expression of the promoter, as provided by a specific Nuclear Factor I (NFI) isoform, NFI-C (Crawford, *et al.* 1998). NFI, because of its inhibitory effects upon PEPCK-C basal, and PKA induced expression, may serve as a means to maintain lowered levels of PEPCK expression, thereby providing an additional level of negative control over hepatic glucose production. NFI binds to the P1 site of the promoter in close proximity to the CRE-1; the results of Crawford, *et al.* (1998), show that NFI-C is capable of inhibiting CREB mediated induction of PEPCK activity *in cyto*. This result is certainly significant if CREB is in fact the *trans*-acting factor which binds to the CRE-1 *in vivo* and mediates cAMP responsiveness.

2.2.3.1.4 Inhibition of PEPCK-C Expression by Insulin

Insulin is a dominant negative inhibitor of basal (Granner, *et al.* 1983), cAMP and glucocorticoid induced (Sasaki, *et al.* 1984 and Granner, *et al.* 1992) expression of PEPCK-C in liver. An insulin responsive sequence (IRS) has been identified in the PEPCK-C promoter in the region from -420 to -402 (O'Brien, *et al.* 1990). The IRS corresponds to the AF2 site present in the glucocorticoid response unit (see section 2.2.3.1.1), making elucidation of its role in PEPCK-C expression difficult, as mutation or deletion of this site effects both the inhibition by insulin, as well as the inductive effects of glucocorticoids (Forest, *et al.* 1990). As mentioned in section 2.2.3.1.1, the IRS is flanked by binding sites for C/EBP and HNF-3, although neither of these sites are particularly similar to consensus. It may be the case that binding of a yet unknown factor to the IRS (an insulin response factor or IRF) may interfere with the subsequent binding of factors to either of these flanking sites, and bring about the inhibitory effects of insulin. However, such a model cannot account completely for the strong dominant negative effects of insulin, as complete deletion of the AF2 site only diminishes the glucocorticoid responsiveness of the PEPCK-C promoter by 50%; a complete inhibition, as is mediated by insulin in the intact promoter, is not observed. Considering these facts, it would seem that insulin likely mediates its effects at various points in the promoter, perhaps also indirectly effecting the DNA binding and or *trans*-activational capabilities of certain transcription factors.

A recent report (Liao, *et al.* 1998) demonstrated that the cAMP and glucocorticoid inducible expression of PEPCK-C could be directly inhibited by activation of a protein

kinase B (PKB/Akt) construct in stable transfected rat hepatoma H4IIE cells. Protein kinase B is a downstream target of a number of phospholipid products of phosphatidylinositol 3-kinase (PI 3-kinase) (Toker, *et al.* 1997), which itself is a downstream component of insulin signal transduction. The inhibition of cAMP and glucocorticoid responses by insulin has previously been shown to be mediated by the activation of PI 3-kinase (Sutherland, *et al.* 1995). The report by Liao, *et al.* (1998), is significant in that it suggests the ability to down-regulate induction of PEPCK-C expression by covalent modification, via PKB, of a variety of factors involved in the glucocorticoid and cAMP responses.

2.2.4 The Effects of *diabetes mellitus* on PEPCK-C Gene Expression

The expression of the cytosolic form of hepatic PEPCK-C is up-regulated in virtually all known syndromes of *diabetes mellitus* (Shrago, *et al.* 1963; Chang, *et al.* 1970; Veneziale, *et al.* 1983 and Nandan, *et al.* 1993). As PEPCK-C is the rate-limiting enzyme of gluconeogenesis, an up-regulation of its activity in liver during diabetes can partially account for the prevailing hyperglycemia which is characteristic of this group of syndromes. In fact, transgenic mice which over-express rat PEPCK-C are observed to be hyperglycemic and resistant to insulin (Valera, *et al.* 1994). However, to date human genetic linkage analysis has shown no association between the loci for PEPCK-C and non-insulin dependant diabetes mellitus (NIDDM) or mature-onset diabetes of the young (MODY) (Vaxillair, *et al.* 1994, Elbein, *et al.* 1995 and Ludwig, *et al.* 1996). Potentially,

the induction of PEPCK-C during diabetes may be due to the increased cAMP/insulin ratio which exists in this state, which thereby results in increased hepatic glucose output (Unger, *et al.* 1970; DeFronzo, *et al.* 1982; Baron, *et al.* 1987 and Consoli, *et al.* 1990). However, work by Friedman, *et al.* (1993), utilizing transgenic mice to determine the regions of the PEPCK-C promoter active in its up-regulation during diabetes, demonstrated that deletion of portions of the CRU had no effect upon the increased expression of the gene during diabetes, nor upon any subsequent down-regulation which could be effected by insulin. However, this work also revealed that diabetic transgenic mice possessing a deletion of the GRU within the PEPCK-C promoter - bovine growth hormone reporter constructs displayed no increase in reporter gene activity, suggesting a requirement of the GRU and glucocorticoids in mediating the up-regulation of PEPCK-C during diabetes. This observation correlates nicely with the classical physiological studies by Long and Lukins, (1936) and by Houssay, (1942), who independently demonstrated the role of glucocorticoids in diabetic hyperglycemia. Although deletion of the GRU in these transgenic mice also disrupts the proposed IRS, interestingly, normoglycemic individuals are still able to respond normally to high carbohydrate, suggesting that this response to carbohydrate is not mediated by insulin acting through the IRS alone, but perhaps through some other site. In fact this particular observation was further supported by the work of Scott, *et al.* (1998) who demonstrated that glucose could directly inhibit PEPCK-C gene expression independently from insulin, potentially through a yet un-characterized carbohydrate responsive element (ChoRE). Carbohydrate responsive elements have been described in a number of other metabolically important genes, including the pyruvate

kinase, and the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase genes (Towle, *et al.* 1997 and Dupriex, *et al.* 1997 respectively).

An obvious short-coming of the work by Friedman, *et al.* (1993), involves the complications brought about by the intrinsic positioning of the IRS within the GRU, such that the observations made with respect to the glucocorticoid responsive regions must also take into consideration the loss of the putative IRS. Given that the IRS can only account for perhaps 50% of the insulin-mediated inhibition of PEPCK-C transcription, its disruption by deletion of the GRU may not significantly downplay the observed requirement of the latter in the diabetic induction of PEPCK-C expression. Despite some short-comings this work does however provide some interesting and novel insight into the molecular mechanisms behind the alterations in PEPCK-C gene expression which occur in diabetes. This aforementioned work has been expanded with use of a less potentially interfering C-reactive peptide reporter construct, again by Friedman, *et al.* (1997), who showed that in genetically obese and diabetic db/db mice, the proximal 460 bp of the PEPCK-C promoter, containing the GRU, is sufficient to mediate its upregulation during diabetes. Furthermore, this work provides supporting evidence regarding the requirement of glucocorticoids to mediate the diabetes-induced changes in expression of not only PEPCK-C, but other gluconeogenic enzymes as well. The results of this research also show that changes in PEPCK-C expression in the diabetic db/db mouse occur independently from events mediated through the IRS.

Thus, the current state of knowledge reveals a vital importance for glucocorticoids in the diabetic induction of PEPCK-C gene expression. Clearly, further investigation into

the *trans*-acting factors which mediate this potentially complicated response will prove invaluable, and may uncover interactions with other hormonal response units which are involved in this complex milieu of metabolic regulation. As a final consideration, even though the role of the IRS has been perhaps down-played in this investigation of diabetes-induced changes in PEPCK-C expression, certainly acknowledgment of the role of insulin in this process cannot be neglected, and it seems likely that further investigation may reveal aspects of its indirect effects at the point of the multiple *trans*-acting factors which are components of the various hormonal response units of the PEPCK-C promoter.

2.3 The CCAAT/Enhancer-Binding Protein (C/EBP)

The CCAAT/Enhancer-Binding protein or C/EBP was first described as a heat-stable DNA-binding protein which was enriched in rat liver nuclei. This *trans*-acting factor demonstrated selective binding to the CCAAT homology sequence of a number of viral promoters (Graves, *et al.* 1986) and to the core homology of many viral enhancers (Johnson, *et al.* 1987). Despite the rationale of naming this protein based on its binding affinity to viral control elements, over the subsequent years C/EBP has been shown to play a pivotal role in mammalian metabolism. C/EBP was first cloned from a rat liver cDNA library by Landschulz, *et al.* (1988a), in the laboratory of Steven L. McKnight. This work laid the foundation for years of further characterization of this transcription factor, and provided the first bits of evidence which led to the discovery of the basic region - leucine zipper (bZIP) DNA-binding - dimerization motif, as well as the Scissors-Grip model of

DNA binding.

2.3.1 The Biochemistry of the CCAAT/Enhancer-Binding Protein

The work of Graves, *et al.* (1986) and Johnson, *et al.* (1987), noted above detected what were initially believed to be unique DNA-binding activities capable of interacting with either the CCAAT homologies of the herpes simplex virus thymidine kinase gene and Moloney murine sarcoma virus (MSV) left terminal repeat, or to the enhancer sequence, 5'-TGTGGTAAG-3' of the MSV, SV40 and polyoma virus promoters. These DNA-binding activities where initially named as the CCAAT binding protein (CBP), and enhancer binding protein - 20 kDa (EBP-20), respectively. Both activities were heat-stable, which facilitated their purification from the majority of other cellular proteins and most DNA-binding activities, and both migrated as 20 kDa species on SDS-PAGE gels. Further study demonstrated that EBP-20 was capable of binding to CCAAT homologies in a manner indistinguishable from CBP, and this was the first indication that the two binding activities were actually arising from a single polypeptide species. Continued work by the McKnight group lead to the production of an antiserum against the CBP or EBP-20 binding activity, and eventually to the isolation of cDNA and genomic clones (Landshultz, *et al.* 1988a). Analysis of the genomic clone revealed that the gene was intronless. Further analysis of the cDNA clone's open reading frame, and examination of rat liver nuclear extracts in the presence of protease inhibitors by Western immunoblotting revealed the true size of the intact polypeptide to be 42 kDa (the initial 20 kDa form was recognized to be

a proteolytic degradation product). The cloned cDNA encoding for a polypeptide of 43-kDa which possessed DNA-binding properties of both CBP and EBP-20, was thereafter referred to as C/EBP or the CCAAT/Enhancer-Binding protein. The DNA-binding activity of C/EBP is localized to a 14-kDa fragment carboxy-terminal fragment, and a 60 amino acid segment within this fragment was observed to have considerable sequence similarity to regions of the Myc and Fos proteins. This conserved amino acid segment was later characterized as the leucine zipper - basic region (bZIP) motif (Landshultz, *et al.* 1988b).

2.3.1.1 The bZIP Domain of C/EBP

Analysis of a 30 amino acid segment within C/EBP's DNA-binding activity revealed the potential for a α-helical conformation of this segment based upon a lack of helix-breaking residues, and an abundance of charged residues. When the primary sequence of this segment was super-imposed upon an idealized helix it revealed the presence of a periodicity of leucine residues, one at every seven residues, spanning over eight helical turns (Landshultz, *et al.* 1988b). The McKnight group demonstrated that the repeating leucine residues were arranged so as to form a hydrophobic strip down one side of the helix (See Figure 2.3). They postulated that two separate molecules possessing such leucine rich amphipathic helices could potentially interact, or dimerize in a manner analogous to a coiled-coil, *via* hydrophobic interactions between their leucine rich hydrophobic strips. This dimerization motif was named the "leucine zipper". Further physical experimentation by McKnight and colleagues showed that the sequence-specific

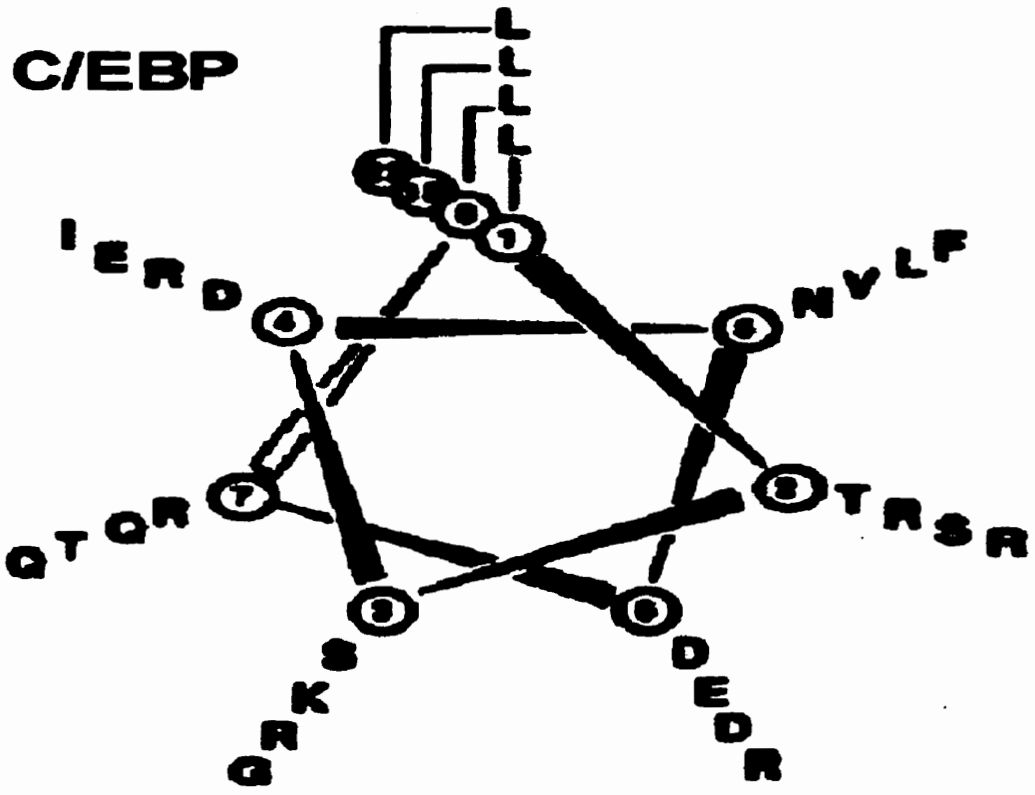

Figure 2.3. Helical Wheel analysis of 28 amino acids of the C/EBP carboxy-terminus known to possess its dimerization capability. Standard one letter amino acid nomenclature is utilized. Most notable is the periodicity of leucine residues (L), corresponding to residues 1, 8, 15 and 22 of the segment analyzed. This strip of hydrophobic leucine residues or "leucine zipper" is proposed to act as a dimerization motif between separate polypeptides possessing similar leucine repeats, presumably *via* hydrophobic interactions. This figure is adapted from Landshultz, *et al.* (1988b).

interaction of C/EBP with DNA required the leucine zipper dimerization interface, as well as a DNA binding surface rich in positively-charged amino acids, which was named the basic region. Contact with DNA was seen to require the presence of the basic regions of two monomers brought into a functional conjunction by the interaction of their leucine zippers, thus the DNA binding domain was observed to be bipartite (Landshultz, *et al.* 1989). The model of dimerization interaction predicted that the two leucine zippers of a potential dimer would dock in an orientation parallel to the dipoles of the helices. Such a manner of interaction would lead to a proximal opposition of the basic regions of the two monomers, such that the two basic regions might have to track around the DNA in opposite directions, thus requiring two half-sites with dyad symmetry in the DNA sequence. This hypothesis was confirmed by further work of this group, upon which they presented the "Scissors-Grip" model of DNA binding (Vinson, *et al.* 1989). Graphical representations of a leucine zipper - basic region motif making DNA contact *via* the "Scissors-Grip" can be seen in Fig. 2.4 and Fig. 2.5. The Scissors-Grip model required that the individual bZIP polypeptides wrap around DNA on the opposite side of the DNA to their initial approach. Such a model allowed for the positively-charged amino acid residues of the basic regions to localize to the edges of the major groove where they would be free to interact with the phosphodiester backbone. The leucine zipper and "Scissors-Grip" model have proven attractive concepts due to their allowance for the formation of heterodimers by polypeptides with similar bZIP domains; such an event allows for a great increase in the potential number of unique hybrid *trans*-activation activities which could make contact with a given dyad symmetric recognition sequence.

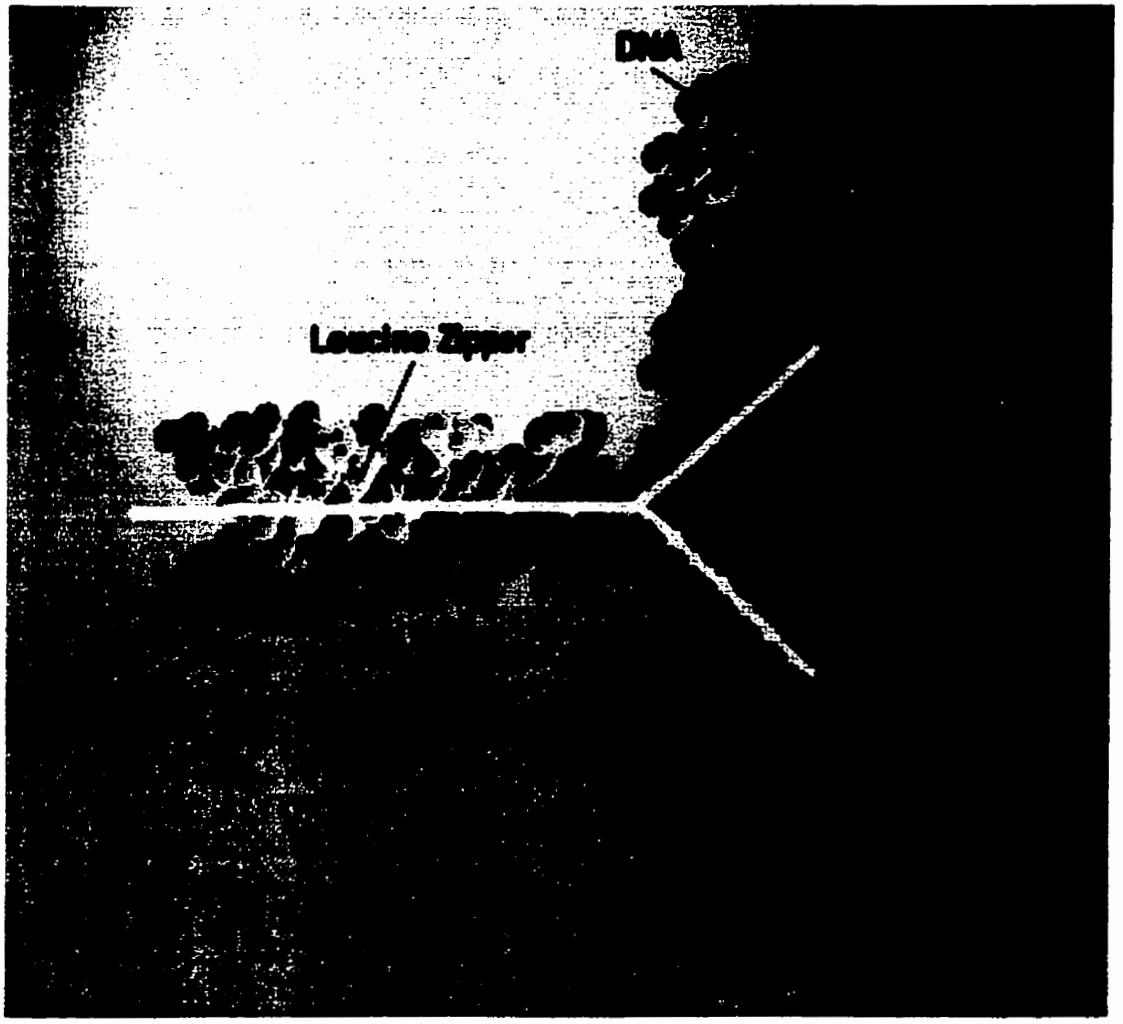

Figure 2.4. A space-filling model representation of a hypothetical leucine zipper - basic region motif making contact with DNA *via* the "Scissors-Grip". The white backbone indicates the position of the dimerized polypeptides, each making contacts with DNA *via* their basic regions. One polypeptide chain is contacting DNA into the plane of the page, the other chain is making contacts out of the plane of the page. This figure is adapted from the "lzip" graphic file found on the http://www.nci.nih.gov/intra/lmb/mnd/pictures.htm web site.

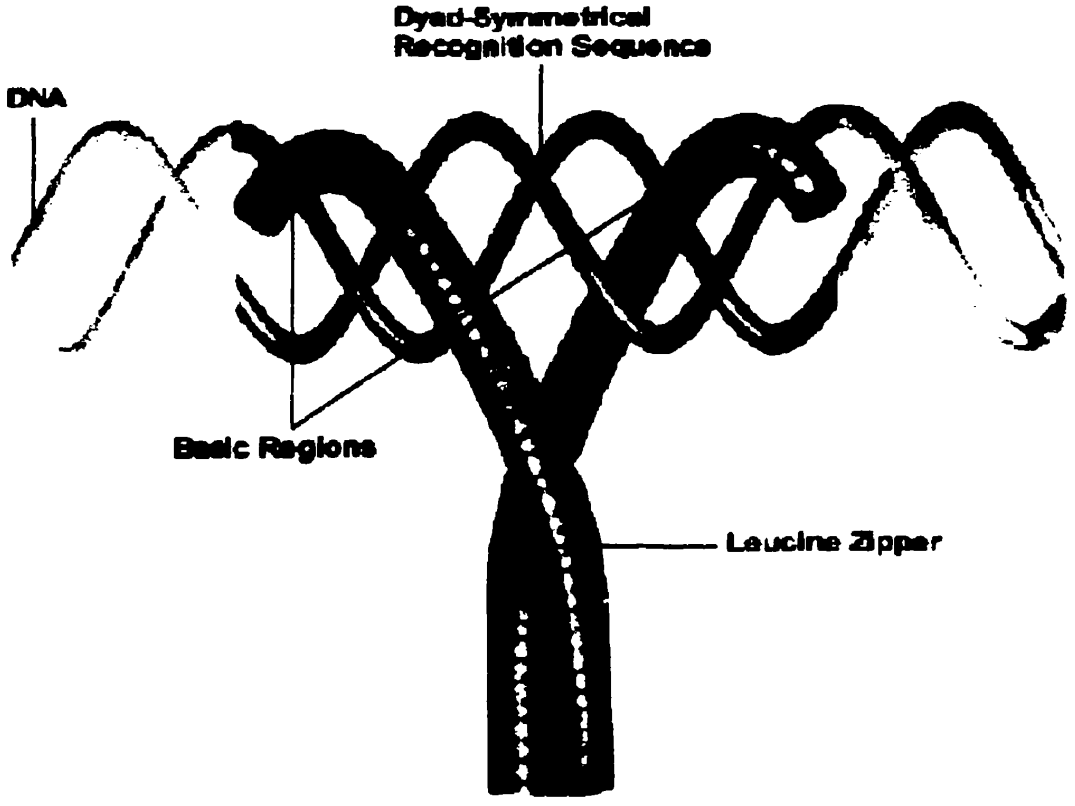

Figure 2.5. Artistic conception of a leucine zipper - basic region motif making contact with DNA *via* the "Scissors-Grip". Dimerization *via* the leucine zipper of each monomer is required for DNA binding by each basic region. The Scissors-Grip model allows the polypeptide chains to wrap around the DNA helix, bringing positively charged residues of the basic regions in contact with the phosphodiester backbone of DNA which is exposed in the major groove. This figure is adapted from "Molecular Zippers in Gene Regulation" by Steven Lanier McKnight, appearing in the April 1991 issue of Scientific American.

Each polypeptide of a hetero-dimer is open to unique regulation over its DNA binding and *trans*-activational capabilities and thus allows for a corresponding increase in the possibilities for regulation of a gene's promoter with which it interacts.

2.3.1.2 The *Trans*-Activation Domains of C/EBP Proteins

The *trans*-activation domains of C/EBPα were first described by Pei and Shih, (1991), who reported that the N-terminus of C/EBP contained three distinct domains; an acidic *trans*-activator domain (amino acids 1 to 107), a proposed attenuator domain (amino acids 107 to 170) and a second, somewhat weaker *trans*-activation domain extending from amino acids 171 to 215 which was rich in proline residues. The nature of C/EBPα's *trans*-activation domains were further investigated by Nerlov and Ziff, (1995), who demonstrated that two amino acid segments termed TE-I (amino acids 1 thru 70) and TE-II (amino acids 70 thru 96) could cooperatively mediate binding to the TATA binding protein (TBP) and TFIIB, showing direct interaction between a transcription factor's *trans*-activational domains and members of the RNA pol II basal transcriptional apparatus. Recently, Roesler, *et al.* (1998), have shown that the cAMP inducibility of the phosphoenolpyruvate carboxykinase promoter is mediated through a region of C/EBPα's *trans*-activational domain lying within its first 124 amino acids. Furthermore, it appears that the region of C/EBPα's *trans*-activation domain which mediates cAMP inducibility is distinct from the regions of its sequence which mediate binding to TBP and TFIIB and its influence upon basal transcription. These facts suggest the possible intervention of a co-activator or

linking factor with C/EBPα, or perhaps interaction with other members of the pol II pre-initiation complex to mediate cAMP inducibility of transcription.

Some brief mention of the unique *trans*-activational domains of C/EBPα's hepatic counterpart C/EBPβ is also in order, as it plays a role in the context of this thesis as well. Initial work by Descombes and Schibler, (1991), localized the C/EBPβ *trans*-activation domain to the first 131 amino acids of the protein. This work was supported by the findings of Trautwein, *et al.* (1995), who demonstrated that the *trans*-activational capabilities of C/EBPβ were located within an acidic region of its sequence, spanning amino acid residues 21 through 105. A sub-region of this acidic domain, spanning amino acids 85 to 95, and which possessed hydrophobic character, was suggested to be the region capable of making contacts with the basal transcription machinery. The work of Williams, *et al.* (1995), expanded further upon the details known of C/EBPβ's *trans*-activation domains. These investigators described two negative regulatory regions in the C/EBPβ primary sequence, RD1 and RD2. RD2 was shown to have cell-specific inhibitory properties, whereas RD1 was seen to be cell-type independent in its inhibitory properties. The presence of these inhibitory domains suggested the requirement for an activation step before C/EBPβ would be available for *trans*-activation. Potentially this activation step could arise from covalent modification of the negative regulatory domains, the nature of the modification either occurring ubiquitously or being cell-type specific depending upon the domain. Furthermore, these researchers were able to subdivide the activation domain of C/EBPβ into three distinct regions or activation domain modules (ADM's). ADM's 2 and 3 were found to be highly conserved amongst C/EBP family members, and ADM3 was

found to share homology to a fos/jun sequence known as homology box 2 or HOB2 (Sutherland, *et al.* 1992). Each activation module was separated by a spacer region of variable length and sequence (S1 and S2). It was observed that independently each ADM had low *trans*-activation capability, however, in combination the three ADM's were able to synergize to provide the full *trans*-activational potential of the transcription factor.

Refer to Figure 2.6 for schematics of the various *trans*-activation, dimerization and DNA-binding domains of C/EBPα and β.

2.3.1.3 Multiple Translation Products of C/EBPα and β

Both C/EBPα and β have multiple translation products which are translated from a single mRNA species by initiating at different AUGs within the same reading frame. It should be stressed that neither of these C/EBP isoforms are understood to undergo differential splicing to produce these translation forms, in fact both genes are intronless. Presumably, multiple translational species can arise from the leaky ribosomal scanning mechanism outlined by Kozak (1989). The phenomenon of multiple C/EBP isoform translation products from a single mRNA was first described for C/EBPβ by Descombes and Schibler, (1991). Two translational forms of C/EBPβ were described, a 39 kDa form, LAP or liver activator protein; and a 20 kDa form, LIP or liver inhibitory protein. These two translation forms share the C-terminal 145 amino acids containing the basic region and leucine zipper motifs, but LIP lacks a *trans*-activation domain. Thus LIP is capable of

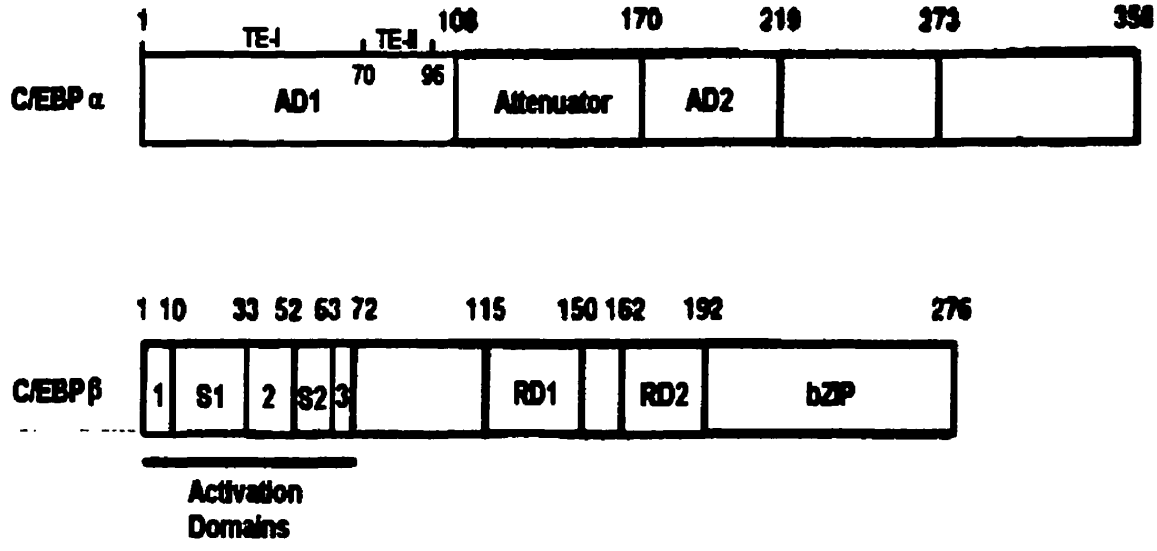

Figure 2.6 Schematic diagram of the *trans*-activation, dimerization and DNA-binding domains of C/EBPα and C/EBPβ. The abbreviations used are: AD, activation domain; bZIP, basic region - leucine zipper; TE, *trans*-activation element; RD, repressor domain; S1 and S2, spacer regions. Numbering corresponds to the amino acid residue of the primary sequence at which a domain begins or ends.

forming homo- or hetero-dimers and thereafter capable of binding DNA, but cannot *trans*-activate a gene promoter. Due to the potential of formation of LAP/LIP heterodimers, these workers proposed a model in which LIP, upon forming a hetero-dimer with LAP, would inhibit its *trans*-activational capability stoichiometrically. Therefore, the relative ratio of LAP/LIP within the cell could determine the overall *trans*-activational activity of C/EBPβ.

Similarly, two different translational forms of C/EBPα have also been reported (Ossipow, *et al.* 1993 and Calkhoven, *et al.* 1994). These two translational forms correspond to the polypeptides C/EBP-42 and C/EBP-30 which differ in their N-termini. Much like LAP and LIP, C/EBP-42 is a full length C/EBPα possessing full *trans*-activational capability, whereas C/EBP-30 lacks a *trans*-activation domain, and is capable of forming transcriptionally inactive heterodimers with full length C/EBPα.

Recently, Lincoln, *et al.* (1998), have presented work challenging the physiological significance of truncated forms of C/EBP proteins, suggesting that these smaller polypeptide species are merely proteolytic degradation products. It should be noted however, that the results of a number of other groups utilizing similar protein lysate preparation conditions suggest that these truncated polypeptides are indeed physiologically significant, and can in fact be influenced by a number of different physiological stimuli, including hormones (Hemati, *et al.* 1998). The multiple translation product phenomenon of C/EBP proteins will be further considered within Section 4 of this thesis.

Mention must also be made of a unique, ubiquitously expressed C/EBP isoform known as C/EBPζ. C/EBPζ is expressed to some degree in all tissues so far examined, and

its expression is induced by DNA damage. C/EBPζ is also known as C/EBP homologous protein (CHOP-10) (Ron and Habener, *et al.* 1992) or GADD 153 (Growth arrest and DNA damage-inducible gene) (Fornice, *et al.* 1989). The C/EBPζ gene spans five kilobases, and unlike most C/EBP isoforms, has four exons. The C/EBPζ protein possesses a leucine zipper and basic region but lacks a *trans*-activation domain. C/EBPζ is capable of forming transcriptionally inactive heterodimers with other C/EBP isoforms, but unlike truncated protein forms of C/EBPα or β, cannot actually bind DNA due to an aberration in its basic region which precludes the formation of a functional DNA-binding domain. Thus, C/EBPζ can also act as an endogenous dominant negative inhibitor of C/EBP isoform activity, but in a manner which completely chelates the heterodimer away from DNA. It should be noted that C/EBPζ is likely not expressed in most cells to any great extent under normal growth conditions. The results of Ron and Habener, (1992), and Fornice, *et al.* (1989), suggest that C/EBPζ expression may be limited to the G_0 stage of the cell cycle.

A similar inhibitory C/EBP isoform, C/EBPγ (Roman, *et al.* 1990; Thomassin, *et al.* 1992 and Cooper, *et al.* 1995), is also thought to be ubiquitously expressed, but its main role appears to be the dominant negative regulation of C/EBP isoform function in undifferentiated, non-proliferating cells.

2.3.2 Tissue-Specific Expression Patterns of C/EBPα and β

The tissue-specific expression of C/EBPα was first described in mouse (Birkenmeier, *et al.* 1989). Considerable levels of C/EBPα mRNA were observed in liver, fat, intestine, lung, adrenal gland, and placenta. These workers also recognized that C/EBPα expression was limited to fully differentiated liver and fat cells, and that in liver and intestine, expression became observable only slightly prior to birth. In humans, C/EBPα was found to be expressed at its highest levels in placenta, although it was also detectable in liver, lung, skeletal muscle, pancreas, small intestine, colon and leukocytes (Antonson, *et al.* 1995).

Prior to the beginning of the work this thesis describes, a few reports had surfaced regarding the hormonal regulation of expression of C/EBP isoforms in adipose tissue, along with a single report concerning regulation in an intestinal cell line. Glucocorticoids were observed to have a negative effect upon C/EBPα expression in fully differentiated 3T3-L1 adipocytes and in white adipose tissue (MacDougald, *et al.* 1994). As will be discussed in Section 4 of this document, and as will be revealed shortly based upon the work of another group, the effect of glucocorticoids upon C/EBPα in adipose tissue is distinct from that of which occurs in liver. Similarly, insulin also down-regulates C/EBPα expression within the fully differentiated 3T3-L1 adipocyte (MacDougald, *et al.* 1995). Potentially also of interest, the known insulin-sensitizing agents, the thiazolidinediones (Forman, *et al.* 1995 and Lehmann, *et al.* 1995) have the completely opposite effect upon C/EBPα expression in the 3T3-L1 adipocyte, inducing both accumulation of mRNA and

protein (Hemati, *et al.* 1998). The only known report of hormonal regulation of C/EBPα in intestinal cells described the inductive effects of serum upon the transcription factor's expression within rat intestinal epithelial IEC-6 crypt cells (Boudreau, *et al.* 1996). Other than the known inhibitory effect upon C/EBPα expression during the acute-phase response in liver (Alam, *et al.* 1992), little has been previously reported of hepatic C/EBPα regulation other than a single report by Ramos, *et al.* (1996), which presented findings demonstrating that the steroid-induced cell cycle arrest of rat hepatoma cells required a glucocorticoid mediated induction of C/EBPα. No other information regarding the hormonal regulation of C/EBPα expression in liver had arisen, thus providing a need for the information which will be presented in Section 4 of this thesis.

A final concept concerning the regulation of C/EBPα expression is that the gene for C/EBPα is auto-regulated by its own gene product. The first evidence of C/EBP auto-regulation was identified in the mouse C/EBP promoter by Christy, *et al.* (1991), and confirmed by Legraverend, *et al.* (1993). These investigators characterized a number of the C/EBP promoter's *cis*-elements, including a potential C/EBP binding site which could form heat-stable complexes with liver-enriched proteins *in vitro.* These liver-enriched factors were recognized by C/EBPα specific antibodies. Further, evidence in support of C/EBP auto-regulation was identified in the human C/EBPα promoter (Timchenko, *et al.* 1995). Although sharing a high degree of sequence similarity with the mouse C/EBP promoter, the human promoter displays a unique mechanism of auto-regulation. No known C/EBP binding *cis*-element occurs in the human promoter, rather auto-regulation occurs *via* the induction of the DNA-binding activity of an ubiquitous factor known as the

upstream stimulatory factor (USF) in response to the α-isoform of C/EBP only.

The cloning, and expression profile of C/EBPβ was reported by independent groups in 1990, and in three different species. The rat isoform of C/EBPβ was initially found to be expressed in liver, lung, spleen, kidney, brain and testes; however, significant protein levels were observed only in liver (Descombes, *et al.* 1990). This was the first indication that post-transcriptional regulation might play an important role in C/EBPβ expression. The rat isoform of C/EBPβ was independently discovered by Poli, *et al.* (1990) and was named IL6-DBP by this group. IL6-DBP was defined as a hepatoma enriched protein sharing strong homology to C/EBP in its leucine zipper and basic region domains, and also characterized by its ability to bind to interleukin-6 (IL-6) responsive elements of a number of acute-phase genes. The human form of C/EBPβ was isolated based upon its ability to bind to the IL-1 response *cis*-element of the IL-6 gene promoter (Akira, *et al.* 1990). This human form of C/EBPβ was termed NF-IL6 or nuclear factor for interleukin-6 expression. Like IL6-DBP, NF-IL6 was seen to be induced by IL-6, as well as IL-1 and LPS, and could bind to the promoters and affect the expression of a number of genes involved in the acute-phase response, inflammation and hemopoiesis. The mouse form of C/EBPβ, AGP/EBP was defined as a C/EBP-like transcription factor which could bind to and effect the transcription of the α_1-acid glycoprotein gene (AGP) (Chang, *et al.* 1990).

Some information regarding the effects of insulin and glucocorticoids upon C/EBP β expression in adipose tissue and intestine has surfaced during the course of this thesis work. In opposition to its effects upon C/EBPα within adipose tissue, insulin has a strong

inductive effect upon C/EBPβ expression (MacDougald, *et al.* 1995). This correlates to some degree to the effects of insulin upon C/EBPβ in liver, which will be presented in Section 4 of this work. Interestingly, in rat intestinal epithelial crypt cells, serum and glucocorticoids both have inductive effects upon C/EBPβ expression (Boudreau, *et al.* 1996). Little information regarding the regulation of C/EBPβ expression in liver has been reported, therefore one objective of this thesis was to present further information on this area. A report by Bosch, *et al.* (1995), suggested that C/EBPβ expression is down-regulated by insulin both *in vitro* and *in vivo*. It also appears that hepatic C/EBPβ expression may be modulated *via* a more ubiquitously expressed transcription factor, the cAMP response element binding protein (CREB) (Niehof, *et al.* 1997). Potentially, induction of C/EBPβ expression *via* mediation by CREB could allow the influence of cellular signals acting through protein kinase A (and thus cAMP). The role of cAMP in the regulation of C/EBPβ expression within liver will be discussed in Section 4.

2.3.3 Biological Roles of C/EBP Isoforms

CCAAT/Enhancer-Binding protein isoforms are a group of related proteins widely involved in tissue differentiation, metabolic regulation and immunity (for minireview see Lekstrom-Himes and Xanthopoulos, 1998). The purpose of this section is to provide an overview of some of the biological processes in which C/EBP isoforms are required, specifically dealing with the α and β isoforms of C/EBP, which are of relevance to this thesis. This overview will begin with a survey of the knowledge attained from *in vivo*

knockout models of C/EBPα and β, followed by a more in depth consideration of the roles of C/EBPα and β in biological processes occurring in adipose tissue, and most relevant to this particular work, in liver.

2.3.3.1 *In Vivo* Knock-Out of C/EBP Isoforms

Homozygous deletion of the *c/ebpα* gene in the mouse was first reported by Wang, *et al.* (1995). The most obvious phenotypic alteration in these C/EBPα knock-out mice was that they succumbed within 8 hours after birth due to hypoglycemia. More detailed analysis of these animals showed that they had no hepatic glycogen stores, and that the mRNA levels of glycogen synthase were reduced. Furthermore, the developmental onset of expression of the two main gluconeogenic genes, PEPCK-C and glucose-6-phosphatase, was delayed and offered a potential explanation for the severe hypoglycemia. Levels of both these enzymes rose back to near control levels over time; however, this required the mice to be sustained *via* glucose gavage. It is possible that some related C/EBP family member might have been compensating for the loss of C/EBPα in the time period after birth, however the fact that these neonates had to be sustained by glucose gavage confounds matters, as both PEPCK-C and glucose-6-phosphatase are potentially regulated by glucose, albeit reciprocally (see section 2.2.4). There was also no accumulation of lipid in the hepatocytes and adipocytes of these animals, and expression of brown adipose tissue uncoupling protein was reduced. Thus, it would appear from this study that C/EBPα is absolutely required for the maintenance of metabolic fuel levels in the neonate, having

profound effects on the normal metabolism of both adipose and liver tissues (reviewed by Darlington, *et al.* 1995). C/EBPα deficient mice were also observed to have defects in their regulation of hepatic growth and lung development (Flodby, *et al.* 1996). The cellular architecture of both hepatocytes and pneumocytes was seen to be perturbed, with liver showing a phenotype similar to a regenerative state, and lung demonstrating abnormal alveolar structure. Hepatocytes were also observed to possesses significantly increased levels of *c-myc* and *c-jun* mRNAs, indicative of active proliferation, as was the observed increase in levels of proliferating cell nuclear antigen/cyclin. In addition to the essential role in maintenance of metabolic homeostasis outlined by the earlier work of Wang, *et al.* (1995), this study also demonstrated a role for C/EBPα in the acquisition of the terminally differentiated state by the hepatocyte. The C/EBPα gene has also been disrupted specifically within the adult mouse liver (Lee, *et al.* 1997*)*, *via* a Cre/loxP-mediated DNA recombination system (Sauer, 1993). The resultant phenotype of hepatic C/EBPα disruption in the adult again showed decreases in expression of PEPCK-C and glycogen synthase, as a well as inhibition of bilirubin UDP-glucuronosyltransferase expression required for proper bilirubin detoxification. As a result these mice became severely jaundiced. The inhibition of PEPCK-C expression in the adult C/EBPα knock-out mouse demonstrates the inability of other C/EBP isoforms to compensate for loss of C/EBPα in the adult, a notable developmental difference from the neonatal knock-out model presented by Wang, *et al.* (1995).

In contrast to C/EBPα disruption, the majority of the phenotypic effects due to the targeted disruption of the C/EBPβ gene appear to be centred upon the immune system

(Tanaka, *et al.* 1995). C/EBPβ deficient mice are extremely susceptible to infection, having impaired macrophage activity. It should be noted, however, that a small subset of C/EBPβ deficient mice demonstrate reduced levels of PEPCK-C expression and a certain percentage of perinatal lethalities, suggesting that C/EBPβ may also play some role in gluconeogenic regulation in the neonate (Croniger, *et al.* 1997).

2.3.3.2 Role of C/EBP Isoforms in the Biology of the Adipocyte

CCAAT/Enhancer-Binding protein isoforms are known to be involved in the differentiation process which converts pre-adipocytes into fat storing adipocytes, as well as being involved in the regulation of genes critical for fat metabolism in the fully differentiated adipocyte (for reviews see MacDougald, *et al.* 1995b; Mandrup, *et al.* 1997 and Darlington, *et al.* 1998). Some of the first evidence regarding the role of C/EBPα in adipocyte differentiation and the *trans*-activation of adipocyte specific genes was presented by Christy, *et al.* (1989). This work demonstrated an up-regulation of C/EBPα expression in the 3T3-L1 pre-adipocyte during differentiation, as well as the ability of this transcription factor to *trans*-activate the promoters of several differentation-induced genes, including the adipocyte-specific fatty acid binding protein 422(aP2) and stearoyl-CoA desaturase 1 (SCD1). The work of Cao, *et al.* (1991), showed that the expression of C/EBPα was up-regulated relatively late within the differentiation process, whereas two other C/EBP isoforms, C/EBPβ and C/EBPδ, were observed to be expressed early on in the differentiation process, and were in fact induced by adipogenic hormones. Expression

of both C/EBPβ and C/EBPδ was seen to drop dramatically just before the induction of C/EBPα later on in the differentiation process. These observations were further supported by the findings of Yeh, *et al.* (1995), who were able to define the individual patterns of C/EBPβ or C/EBPδ induction by specific adipogenic hormones. These workers were able to demonstrate that these two C/EBP isoforms were responsible for relaying certain adipogenic hormonal signals, ultimately resulting in the activation of C/EBPα, which is required for the *trans*-activation of adipose-specific genes involved in uptake, synthesis, and storage of fatty acids. Of importance to this thesis, two separate groups were able to demonstrate the absolute requirement for C/EBPα in the differentiation and metabolism of the adipocyte, by utilizing antisense construct methodology (Samuelsson, *et al.* 1991 and Lin and Lane, 1992). Both groups were able to demonstrate that expression of specific C/EBPα antisense constructs could block expression of both C/EBPα mRNA and protein, resulting in the inhibition of a number of adipocyte-specific genes and an inability of the fat cell to accumulate triacylglycerides.

Not surprisingly, C/EBPα is also known to *trans*-activate a number of adipocyte-specific genes involved in glucose metabolism and global energy homeostasis. For example, C/EBPα is thought to be involved in the tissue-specific and metabolic regulation of the insulin-responsive glucose transporter (GLUT4) (Kaestner, *et al.* 1990). The α-isoform of C/EBP is also thought to be an important regulator of leptin, the *obese* gene product (He, *et al.* 1995; Hwang, *et al.* 1996 and Hollenberg, *et al.* 1997). An additional level of complexity has been observed, concerning the ability of C/EBPα and C/EBPδ to

trans-activate the promoter for the γ_2 isoform of the peroxisome proliferator activated receptor (PPAR γ_2) (Clarke, *et al.* 1997), a form of nuclear receptor involved in regulating adipogenesis and lipid metabolism. As is the case for C/EBPα, PPAR γ_2 is also involved in *trans*-activating the leptin gene.

Thus, C/EBP isoforms are deeply entwined in the regulatory mechanisms of adipocyte differentiation and metabolism, serving at multiple levels of regulation and at various control points in order to insure the appropriate development and maintenance of the biological profile of the adipocyte.

2.3.3.3 Role of C/EBP Isoforms in the Biology of the Hepatocyte

Within the liver cell, C/EBP isoforms appear to play a part not only in the *trans*-activation of a number of metabolically vital genes, but also hold important roles in immunity, liver cell proliferation, and regeneration.

In the case of regulation of inflammatory and immunity functions (for minireview see Poli, 1998), C/EBPα does not seem to play a major role. In fact, in response to inflammatory stimuli, C/EBPα expression is down-regulated with a subsequent upregulation of the β and δ isoforms of C/EBP (Alam, *et al.* 1992). Both C/EBPβ and C/EBPδ are capable of *trans*-activating the promoters of various genes involved in immunity, including the cytokines IL-6, IL-1β, tumor necrosis factor - α (TNF-α), IL-8 and IL-12 (Akira, *et al.* 1990; Wedel, *et al.* 1996; Pope, 1994 and Shirakawa, *et al.* 1993), and various genes involved in cell mediated immunity such as nitric oxide synthase

(Lowenstein, *et al.* 1993), lysozyme (Goethe, *et al.* 1994) and granulocyte colony-stimulating factor (Dunn, *et al.* 1994).

The concept that C/EBPα played an antiproliferative role in differentiated cell types was first suggested by Umek, *et al.* (1991). This idea was confirmed in the liver cell by Mischoulon, *et al.* (1992), who were able to demonstrate a decrease in C/EBPα mRNA levels correlated with the regenerative response of the cells after a partial hepatectomy. Further work revealed that not only the expression of C/EBPα, but also its DNA-binding activity, was down-regulated in the regenerating liver (Rana, *et al.* 1995). In contrast, the DNA-binding ability of the β-isoform of C/EBP was seen to increase under proliferative conditions, although no change in its overall expression was witnessed. These concepts were supported by the work of Diehl, *et al.* (1996), which showed that in a rat hepatocyte cell line, which normally expressed minimal amounts of C/EBPα, adenoviral-mediated overexpression of this same factor had a dominant antiproliferative effect.

Much like in adipose, C/EBP isoforms are involved in the *trans*-activation of a considerable number of metabolically relevant genes in the hepatocyte, including acetyl-CoA carboxylase (Tae, *et al.* 1995), serum albumin (Friedman, *et al.* 1989), insulin responsive GLUT-4 (Kaestner, *et al.* 1990) and most importantly for the purposes of this thesis, PEPCK-C (for review see Croniger, *et al.* 1998).

C/EBP isoforms are known to bind to multiple sites of the rat PEPCK-C promoter, and assist in the mediation, and potential cross-talk between a variety of hormonal responses (see section 2.2.3.1). Given that individual C/EBP isoforms have unique expression patterns and responses to hormonal regulation in liver (as will be revealed in

section 4), and can potentially heterodimerize as well, the identity of C/EBP isoforms absolutely required for individual hormonal responses of the PEPCK-C promoter is of great interest. Along with characterization of the hormonal regulation of the two main hepatic C/EBP isoforms, C/EBPα and C/EBPβ, the other primary aim of this work is to assess the C/EBP isoform requirements in mediation of several PEPCK-C promoter hormonal responses, including those of glucagon (*via* cAMP) and glucocorticoids.

2.4 Approaches for Inhibition of C/EBP Isoform Activity or Expression

Two of the most widely utilized methods for inhibiting the activity of a given cellular factor are the use of the dominant negative inhibitor to interfere with the factor at the level of its biochemical activity, and the use of antisense constructs to inhibit the expression of the factor within the intact cell.

2.4.1 GBF-F, a Dominant Negative Inhibitor of C/EBP Isoform Activity

The concept behind producing a dominant-negative molecule to inhibit C/EBP isoform function arose due to the development of an inter-helical salt bridge rule to describe the specificity of dimerization between two helices of a potential leucine zipper (Vinson, *et al.* 1993). The inter-helical salt bridge rule is based upon the known dimerization specificities, in terms of electrostatic interactions, of a number of bZIP family members. These guidelines allow for considerable success in the prediction of potential

heterodimerization partners based upon the identity of amino acids at given positions of the amphipathic helix. Using this rule, it is possible to design specific polypeptides capable of heterodimerizing with a specific bZIP-containing monomer. The manufacture of these polypeptides could potentially result in the production of a dominant-negative molecule if the designed polypetide lacks a *trans*-activation domain, or in some manner interferes with the formation of a proper "Scissors-Grip" necessary for DNA-protein interaction.

A chimeric dominant-negative molecule to C/EBP isoforms has been created utilizing the inter-helical salt bridge guidelines (Olive, *et al.* 1996). This molecule known as GBF-F, consists of the DNA binding - basic region of the plant bZIP protein GBF-1 (Giuliano, *et al.* 1988 and Schindler, *et al.* 1992) fused to an engineered leucine zipper known as the "F" zipper (Vinson, *et al.* 1993). The "F" zipper preferentially forms heterodimers with C/EBP isoforms due to specific amino acid substitutions made at positions e and g of the zipper amphipathic helix. Thus the GBF-F molecule, by preferentially forming heterodimers with C/EBP family members, inhibits C/EBP *trans*-activation due to the inability of the GBF basic region to recognize the dyad symmetrical C/EBP consensus *cis*-element. It should be noted that GBF-F may potentially form *trans*-activationally inactive heterodimers with fos and jun monomers (C.R. Vinson, personal communication to W.J. Roesler). The dominant-negative nature of GBF-F over C/EBP isoforms makes it an excellent tool for ascertaining the overall necessity of C/EBP isoforms in the transcriptional responsiveness of a given gene. The GBF-F molecule has been utilized in a portion of this thesis work to determine the need for C/EBP isoforms in

the hormonal responses of the rat PEPCK-C promoter. Obviously, GBF-F cannot distinguish between individual C/EBP family members, due to the similarity of the leucine zippers among isoforms. The following section (2.4.2) will address the use of specific antisense constructs to inhibit either C/EBPα or C/EBPβ expression within the intact cell.

2.4.2 The Utilization of Antisense RNA Constructs to Inhibit Gene Expression

Antisense RNAs interfere with gene expression by hybridizing to target RNA, rendering it functionally inactive, the target RNA being the complimentary sense mRNA of the gene of interest (for reviews see Takayoma, *et al.* 1988; van der Krol, *et al.* 1988; Wagner, 1995 and Branch, 1998). The manner in which an antisense molecule inhibits the expression of a given gene can be varied, but in any event its purpose is to ultimately inhibit the translation of the target mRNA after formation of a sense-antisense hybrid RNA. The formation of this duplex hybrid may render it more susceptible to double strand-specific nucleases, or may potentially interfere with post-transcriptional processing of the sense transcript.

Antisense molecules have been utilized to inhibit the expression of a variety of eukaryotic genes, including as examples, thymidine kinase (Izant, *et al.* 1984 and Kim and Wold, 1985), the *c-myc* proto-oncogene (Yokoyama and Imamoto, 1987), *c-fos* (Ledwith, *et al.* 1990), growth hormone (Paulssen, *et al.* 1990) and the glucocorticoid receptor (Pepin and Barden, 1991). As mentioned in section 2.3.3.2, two groups have utilized C/EBP antisense constructs to assess its roles in adipocyte differentiation and adipocyte specific

gene expression. The construct utilized by Samuelsson, *et al.* (1991), in 3T3-F442A pre-adipocytes corresponded to full-length C/EBPα and produced a 62% decrease in C/EBPα mRNA levels, whereas the construct utilized by Lin and Lane (1992), corresponded to an approximately 400 bp segment complementary to the coding region of the C/EBPα *trans*-activation domain. Lin and Lane's antisense RNA, perhaps because of its smaller size, produced a much more effective 95-98% reduction in C/EBPα mRNA levels, and essentially abolished C/EBP protein levels within the stable transfected 3T3-L1 pre-adipocyte. The work presented in this thesis has utilized C/EBP antisense constructs similar to those utilized by Lin and Lane (1992). These antisense constructs, specific for either C/EBPα or C/EBPβ, are complimentary sequences corresponding to regions of either protein's *trans*-activation domain. The sequences utilized are specific for each individual isoform, and thus show specificity of targeting for the intended isoform.

Clearly, the use of antisense methodology is a powerful tool in the characterization of the roles of specific gene products within the intact cell as long as special attention is payed to the uniqueness of the sequences utilized, as well as to their size relative to the full length transcript.

2.5 Objectives

The objectives of this thesis are two-fold and are based upon the inadequacy of the current literature to define the hormonal regulation profiles of C/EBP isoforms in the liver, as well as the need to identify the specific C/EBP isoforms which participate in the complex hormonal responses of the endogenous gene for PEPCK-C in the hepatocyte.

As presented in section 2.3.2, a significant amount of information has arisen concerning the hormonal regulation of C/EBP isoforms in metabolically relevant tissues such as adipose and intestine. However, information regarding the patterns of hormonal regulation within the hepatocyte are lacking. As this literature review has attempted to demonstrate, C/EBP isoforms are vital for the expression and regulation of many metabolically vital genes, including many which are involved directly in glucose homeostasis within the liver. As many levels of regulation are required in the complex milieu of metabolic processes, it should not be surprising that a higher order of hormonal control over actual gene regulatory factors should occur, and that these regulatory mechanisms should be uniquely tailored to the specific tissue in which they operate.

The gene promoter for PEPCK-C is an excellent model for the study of hormonal regulation of gene expression, given its multiple integrated responses to hormones, and the complex organization of its promoter architecture. A considerable amount of work has been done towards understanding the mechanisms of hormonal regulation of hepatic PEPCK-C expression. However, much of this work regarding the roles of C/EBP isoforms has been based upon synthetic or artificial systems, and although providing an excellent framework for understanding, cannot by its nature truly reflect the mechanisms which

operate in the intact cell upon the endogenous gene promoter. Given that individual C/EBP isoforms have unique patterns of expression, and hormonal regulation within the liver, along with their inherent ability to form heterodimers which can produce multiple forms of uniquely regulated *trans*-acting factors, the need to ascertain the exact requirements for specific C/EBP isoforms in mediation of these hormonal response mechanisms should be obvious.

Given these above considerations, the specific objectives of this thesis are:

(1) To define the patterns of regulation of various hormones over the expression of the two main liver C/EBP isoforms, C/EBPα and C/EBPβ, within rat hepatoma cells, as well as some characterization *in vivo*, in adult rat liver.

(2) To define and characterize which specific C/EBP isoforms mediate the various hormone responses of the rat PEPCK-C promoter in liver. C/EBP isoform requirements will be characterized by the production and analysis of a number of rat hepatoma H4IIE stable cell lines expressing the dominant-negative inhibitor GBF-F, or constructs designed to produce specific C/EBPα or C/EBPβ antisense RNAs.

3. MATERIALS AND METHODS

3.1 Reagents

All reagents used were of molecular biology or analytical grade as appropriate. The names of the reagents utilized and the name of their supplier are listed in Table 3.1. The addresses of the individual bio-molecular supply companies from which reagents were obtained are listed in Table 3.2.

Table 3.1 A List of Reagents Utilized

Reagent	Supplier Name
Common Reagents	
Absolute Ethanol	BDH
Acrylamide	Bio-Rad
AG® 501-X8 20-50 mesh Analytical Grade Mixed Bed Resin	Bio-Rad
Agarose	Bio-Rad
Ammonium Persulphate	Bio-Rad
Bovine Serum Albumin	ICN
Bovine Serum Albumin (RNAse Free)	Pharmacia
Bromophenol Blue	Sigma
Coomassie® Brilliant Blue Stain	Sigma

Calcium Chloride ($CaCl_2$)	BDH
Diethylpyrocarbonate (DEPC)	BDH
Dimethylsulfoxide (DMSO)	Sigma
Dithioerythritol (DTE)	Sigma
Dithiothreitol (DTT)	Promega
Ethidium Bromide	Sigma
Ethylene-Diamine Tetraacetic Acid Disodium Salt (EDTA)	BDH
Ficoll 400	Pharmacia
Formamide	BDH
Glacial Acetic Acid	BDH
Glycine	BDH
N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid (HEPES)	USB
Isobutanol	BDH
Isopropanol	BDH
LMP (low melting-point) - Agarose	Bio-Rad
Magnesium Chloride ($MgCl_2$)	BDH
2-Mercaptoethanol (2-ME)	Sigma
Methanol	BDH
N,N'-Methylene-Bis-Acrylamide	Bio-Rad
(3-[N-Morpholino]propanesulfonic acid) MOPS	Sigma
Nonidet NP-40	BDH
Phenol:Chloroform (1:1) (Molecular Biology Grade)	Sigma

1,4 - Piperazine Diethane Sulfonic Acid, 1.5 Sodium Salt (PIPES)	Sigma
Polyethylene glycol 8000 (PEG 8000)	BDH
Sephadex™ G-50 - Fine DNA Grade	Pharmacia
Sodium Acetate	BDH
Sodium Bicarbonate ($NaHCO_3$)	Fischer
Sodium Citrate	BDH
Sodium Dodecyl Sulphate (SDS)	Pharmacia
Spermidine-3HCl (N-[3-Aminopropyl]-1,4-Butanediamine)	Sigma
N,N,N',N'-Tetramethylenediamine (TEMED)	Sigma
Tris-[hydroxymethyl] Aminomethane (Tris)	GIBCO-BRL
Trypsin-EDTA	GIBCO-BRL
Tween-20	Sigma
Urea	BDH
Xylene Cyanol FF	Sigma
Yeast Total RNA	Boehringer Mannheim
Reagents for Bacterial Growth	
Ampicillin	ICN
Bacto-Agar	DIFCO
Bacto-Tryptone	DIFCO
Bacto-Yeast Extract	DIFCO

Cell Culture Reagents	
Calf Serum (USA)	GIBCO-BRL
Cell Culture Freezing Medium (DMSO)	GIBCO-BRL
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) - with Glutamine	GIBCO-BRL
Fetal Bovine Serum (Mexican)	GIBCO-BRL
Hormones and Second Messengers	
8-Chlorophenylthio - Cyclic Adenosine Monophosphate (8CPT-cAMP)	Sigma
Dexamethasone	USB
Insulin (Porcine Pancreas)	Sigma
NPH-Insulin	Connaught Laboratories
Miscellaneous	
5L Chemstrips	Boehringer Mannheim
Gentamicin Sulphate (G418) (Genticin)	GIBCO-BRL
Streptozotocin	Sigma
Theophylline (1,3-Dimethylxanthine)	Sigma
TRIzol™ Reagent	GIBCO-BRL
Enzymes	
DNA Ligase	GIBCO-BRL
DNA Polymerase I, Klenow Fragment - Labelling Grade	Promega

DNAse I (10U/µl)	Boehringer Mannheim
RNA Guard® - Placental RNAse Inhibitor	Pharmacia
RNAse A	Boehringer Mannheim
RNAse T_1	Boehringer Mannheim
RQI DNAse (1U/µl)	Promega
Taq DNA Polymerase - from *Thermophilis Aquaticus*	GIBCO-BRL
T4 DNA Ligase	GIBCO-BRL
T7 RNA Polymerase	Pharmacia
Reagents for Hybridization Protocols	
Deoxyribonucleic Acid (from salmon sperm)	Sigma
Dextran Sulphate - Sodium Salt	Sigma
GeneScreen Plus® Hybridization Transfer Membrane	NEN-Mandel
Hexanucleotide Mix	Boehringer Mannheim
Polyvinylpyrrolidone (PVP)	BDH
Reagents for Protein Protocols	
Aprotinin	Sigma
Bio-Rad Blotting Grade Blocker Non-Fat Dry-Milk	Bio-Rad

Bio-Rad Protein Assay Dye Reagent	Bio-Rad
Bio-Rad Trans-blot Transfer Medium (Nitrocellulose)	Bio-Rad
Goat Anti-Rabbit Conjugated Horse Radish Peroxidase Secondary Antibody	Bio-Rad
Pepstatin	Sigma
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
Rabbit Anti-C/EBPα Primary Antibody	Santa Cruz Biotechnology
Rabbit Anti-C/EBPβ Primary Antibody	Santa Cruz Biotechnology
Rabbit Anti-TFIIEα Primary Antibody	Santa Cruz Biotechnology
Renaissance® Chemiluminescence Reagent	NEN-Mandel
Sodium Deoxycholate	Sigma
Sodium Fluoride (NaF)	Sigma
Sodium Orthovanadate	Sigma
Nucleotides	
2'-Deoxyadenosine 5'-Triphosphate (dATP)	Pharmacia
2'-Deoxycytosine 5'-Triphosphate (dCTP)	Pharmacia
2'-Deoxyguanosine 5'-Triphosphate (dGTP)	Pharmacia
2'-Deoxythymidine 5'-Triphosphate (dTTP)	Pharmacia
[α-^{32}P] 2'-Deoxycytosine 5'-Triphosphate (3000 Ci/mmol)	NEN-Mandel
[α-^{35}S] 2'-Deoxyadenosine 5'-Triphosphate (500 Ci/mmol)	NEN-Mandel
2' 3'-Dideoxyadenosine 5'-Triphosphate (ddATP)	Pharmacia

2' 3'-Dideoxycytosine 5'-Triphosphate (ddCTP)	Pharmacia
2' 3'-Dideoxyguanosine 5'-Triphosphate (ddGTP)	Pharmacia
2' 3'-Dideoxythymidine 5'-Triphosphate (ddTTP)	Pharmacia
[α–^{32}P] Uridine-5'-Triphosphate (3000 Ci/mmol)	NEN-Mandel

Table 3.2 The Names and Addresses of Reagent Suppliers

Supplier	Address
BDH	British Drug House, Saskatoon, Saskatchewan, Canada
Bio-Rad	Bio-Rad Laboratories, Mississauga, Ontario, Canada
Boehringer Mannheim	Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada
Connaught Laboratories	Connaught Laboratories, Willowdale, Ontario, Canada
DIFCO	DIFCO Laboratories, Detroit, Michigan, USA
GIBCO-BRL	Bethesda Research Laboratories, Burlington, Ontario, Canada
ICN	ICN Biomedical Canada Ltd., St. Laurent, Quebec, Canada
NEN - Mandel	Mandel Scientific Company, Guelph, Ontario, Canada

Pharmacia	Pharmacia LKB Biotechnology Ltd., Baie d'Urfe, Quebec, Canada
Promega	Fisher Scientific, Nepean, Ontario, Canada
Santa Cruz Biotechnology	Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA
Sigma	Sigma Chemical Co., St. Louis, Missouri, USA
United States Biochemical (USB)	United States Biochemical Corporation, Cleveland, Ohio, USA

3.2 Bacterial Strains and Media Preparation

A single *Escherichia coli* strain, *E. coli* NM522, was utilized for all cloning manipulations conducted during the course of this thesis work. This NM522 strain has been described by Gough and Murray (1983) and Woodcock, *et al.* (1989).

Bacterial cells which were plated were propagated on Luria-Bertani medium (LB) plates at 37 °C. LB plates were prepared by combining water, 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl and 15 g of bacto-agar in a volume of 1 L. This mixture was autoclaved for 20 min at 15 lb/sq. in. Ampicillin was added into the medium at a concentration of 50 μg/mL once it was sufficiently cooled, the plates were then poured.

Terrific broth (Sambrook, *et al.* 1989) was used to propagate bacterial cells in a liquid medium at 37 °C. Terrific broth was prepared by combining water, 12 g of bacto-tryptone, 24 g of bacto-yeast extract and 4 mL of glycerol in a volume of 900 mL. This

mixture was autoclaved for 20 min at 15 lb/sq. in. After sufficient cooling, 100 mL of a sterile solution containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added aseptically. This solution of potassium salts was prepared by combining 2.3 g of KH_2PO_4 and 12.54 g of K_2HPO_4 per 100 mL of sterile water and autoclaving at 15 lb/sq. in. for 20 min.

3.3 Cell Culture of Rat Hepatoma H4IIE Cells

Rat hepatoma H4IIE cells (Reuber, 1961 and Pitot, *et al.* 1964) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1) - with glutamine, containing 5% calf serum and 5% fetal bovine serum, at 37 °C and 5% CO_2. All cell lines were passaged twice a week via trypsinization at a 1:10 dilution. All cell lines were maintained on 100 mm tissue culture plates. Stable transfected cell lines were selected in media containing 400 μg/mL gentamicin sulphate (G418). After individual clones had been isolated, the selection pressure was reduced and a medium containing 100 μg/mL G418 was utilized.

Liquid nitrogen cell stocks were prepared by trypsinizing plates and resuspending the detached cells in 10 ml of cell media. The resuspended cells were then pelleted by centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant removed and the cells resuspended in 1 mL of cell stock freezing media containing fetal bovine serum, calf serum and 10 % DMSO.

3.4 Animal Handling and Experimental Manipulations

Male Sprague-Dawley rats of approximately 150-200 g weight were utilized for two sets of experiments which will be further outlined in section 4. Principles of laboratory animal care were adhered to and all protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply. Animals were allowed to feed on Purina rat chow *ad libitum* and were maintained on a 12 h day-night cycle.

Animals which were subjected to a cAMP bolus were initially injected with the cAMP phosphodiesterase inhibitor theophylline (30 mg per kg body weight) in a sterile PBS vehicle. Following this initial injection animals were injected with 8-CPT-cAMP also in PBS vehicle at a dose of 30 mg per kg body weight at 30 min intervals over a 90 min time period. All control animals received PBS injections at the same time points. Following these injections, animals were killed by decapitation and a portion of liver was quickly frozen in liquid nitrogen and stored at -80 °C until RNA isolation could take place (see section 3.7). An additional portion of liver was immediately homogenized in radio-immuno-precipitation assay (RIPA) buffer for protein lysate preparation (see section 3.13), protein lysates were stored at -80 °C until they were analysed.

Animals were made diabetic by an intra peritoneal injection of streptozotocin (80 mg per kg body weight) in 100 mM sodium citrate [pH 4.0] and 150 mM NaCl vehicle. Control animals were injected with vehicle only. All animals were tested for glucosuria after 5 days with 5L Chemstrips, and all streptozotocin-treated animals tested positive and displayed weight loss in excess of 20 g. At this point half of the diabetic animals were started on daily injections of 5 IU of NPH insulin injected subcutaneously for 5 days and

were monitored with Chemstrips to insure appropriate insulin replacement. After 5 days the animals were killed and livers isolated. Untreated diabetic animals were killed by decapitation 5 days after testing positive for glucosuria and control animals were also killed at this time. A portion of liver was freeze-clamped in liquid nitrogen and then stored at -80 °C until total RNA isolation could take place, while another portion of liver was immediately homogenized in RIPA buffer for protein lysate preparation. At the time of death all untreated diabetic animals presented blood glucose levels in excess of 25 mM.

3.5 General Molecular Cloning Procedures

In general, all protocols outlined within this section are at least partially based upon those presented in Sambrook, *et al.* (1989).

3.5.1 The Polymerase Chain Reaction (PCR)

Polymerase chain reactions were assembled in a 50 μL volume containing filter sterile water, 1X PCR buffer containing 20 mM Tris-HCl [pH 8.4] and 50 mM KCl; 400 μM of each dNTP, a mix of forward and reverse PCR primers (20 pmole each), 1.5 mM $MgCl_2$, approximately 1 μg of template DNA and finally, approximately 2.5 units of *Taq* DNA polymerase. The PCR reaction was topped off with 20 μL of mineral oil to prevent evaporation. Thermocycling occurred in a Perkin Elmer Cetus DNA Thermal Cycler utilizing the following program: one denaturing cycle at 94 °C for 3 min followed by 35

cycles of denaturing at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 60 sec. These cycles were followed by a 7 min extension at 72 °C. After thermocycling PCR products were analysed by agarose gel electrophoresis (section 3.5.2).

3.5.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was typically carried out on 1% agarose gels in 1X TAE buffer. Ethidium bromide was added to the agarose solution to a concentration of 0.5 μg/mL. The running buffer used for agarose gel electrophoresis was 1X TAE, containing 40 mM Tris-acetate and 1 mM EDTA [pH 8.0]. Enzyme digests or PCR reactions were terminated by adding in an appropriate volume of 5X agarose gel sample buffer containing 50% glycerol, 50 mM EDTA [pH 8.0] and traces of bromophenol blue and xylene cyanol FF, and then placing the mixture on ice. Agarose gels were electrophoresed at between 90 and 110 volts, until the appropriate amount of band resolution had been achieved.

3.5.3 Gel Fragment Isolation and Ligation Reactions

After electrophoresis a small section of gel distal to the DNA band of interest was excised and filled with molten 0.9% low melting-point (LMP)-agarose which was then allowed to gel. Following this the gel was again electrophoresed at 90 volts, constant voltage, for an appropriate amount of time in order to insure the band of interest had entered the LMP-agarose. The band was then excised in the smallest volume of agarose

possible. The DNA band of interest was then purified away from the LMP-agarose using the Geneclean® protocol as according to the manufacturers instructions. Purified plasmid DNA was eluted from the final washed Glassmilk®-DNA pellet into sterile water by incubation at 65 °C for 10 min and with frequent vortexing.

Ligations were generally set up with a 3:7 (v:v) ratio of vector : insert. Ligation reactions were prepared in a 20 μL volume, thus 3 μL of purified vector DNA and 7 μL of purified insert DNA were added, along with T4 DNA Ligase (1 unit, corresponding to approximately 300 cohesive-end ligation units), T4 DNA Ligase buffer containing 50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (w/v) PEG-8000; and finally sterile water to bring the reaction up to volume. Ligations were allowed to proceed at 16 °C overnight and were then terminated by heating at 65 °C for 10 min.

3.5.4 Preparation of Competent *E. coli* NM522 and Transformation

To prepare competent cells, a single colony of *E. coli* NM522 was used to innoculate 50 mL of LB media, which was then allowed to grow at 37 °C overnight with shaking. The next morning, 4 mL of this saturated culture was used to innoculate a 400 mL culture of LB medium which was then incubated at 37 °C with shaking until a density of growth indicated by an OD_{590} of 0.375 had been reached. Upon reaching this proper stage of growth, the culture was aliquoted into pre-chilled sterile tubes and allowed to incubate on ice for 10 min. The bacterial cells were then pelleted by centrifugation at 3000 rpm for 7 min at 4 °C. The supernatant was decanted and the cells resuspended in 10 mL

of ice-cold $CaCl_2$ solution containing 60 mM $CaCl_2$, 15% glycerol and 10 mM PIPES [pH 7.0]. The cells were again pelleted by centrifugation at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and the cells resuspended in 10 mL of ice-cold $CaCl_2$ solution and then incubated on ice for 30 min. After this incubation, cells were centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and the final competent cell pellet was resuspended in 2 mL of ice-cold $CaCl_2$ solution. Competent cells were stored in aliquots at -80 °C until needed.

For transformation of competent cells, 10 µL of the appropriate ligation reaction (see section 3.5.3) was added to 100 µL of competent NM522 cells in a 12 mL polypropylene tube and incubated on ice for at least 30 min. After this incubation, transformation was achieved by heat-shocking the cells at 42 °C for 2 min. The heat-shocked cells were then plated on LB medium containing 50 µg/mL ampicillin and allowed to grow overnight at 37 °C. Single colonies were picked for small scale plasmid isolations and subsequent diagnostic analysis.

3.5.5 Small Scale Plasmid Isolations

All plasmids were replicated within *E. coli* strain NM522 (see section 3.2). A stab of a single bacterial colony was used to innoculate a 5 mL culture of Terrific broth containing 100 µg/mL ampicillin. Cultures were allowed to incubate at 37 °C with shaking overnight. The protocol utilized for small scale plasmid isolation is based upon the method of Lee and Rasheed (1990). The final pellet containing purified plasmid DNA was

dissolved in 25 μL of TE buffer. A typical plasmid isolation using this procedure would yield approximately 20 to 35 μg of plasmid DNA from a 1.5 mL bacterial culture.

3.5.6 Sequencing of Double-stranded Plasmid DNA

The protocol utilized for double-stranded sequencing of plasmid DNA is based upon that presented by Akella and Porter (1993), which in turn is based upon the original dideoxy-nucleotide sequencing method of Sanger (1977). Reactions were terminated by the addition of 4 μL of denaturing polyacrylamide gel sample buffer containing 80% formamide, 10 mM EDTA [pH 8.0] and traces of bromophenol blue and xylene cyanol FF, and incubating on ice.

A denaturing 6% polyacrylamide (19:1 acrylamide : N,N'-methylene-bis-acrylamide) 8 M urea gel was used for resolving sequencing reactions. The sequencing gel was cast in a Kodak IBI sequencing gel apparatus. A shark's-tooth comb was used for sample application. Sequencing reactions were denatured by heating at 80 °C for 4 min and were then placed on ice; approximately 4 μL of each reaction was applied to the gel per lane. The running buffer used for electrophoresis was 0.5X TBE, containing 45 mM Tris-borate and 1 mM EDTA [pH 8.0]. Gels were run at 2000 volts until the appropriate amount of resolution was achieved. Sequencing gels were dried in a vacuum gel drier before overnight autoradiography at room temperature using Kodak X-OMAT film.

3.5.7 Large Scale Plasmid Isolations

Bacterial cultures for large scale plasmid isolations were inoculated by first streaking a Luria-Bertani (LB) plate containing 50 μg/mL ampicillin with culture from a -80 °C DMSO stock and incubating overnight at 37 °C. A single colony stab from this streaked plate was used to innoculate a 5 mL culture of terrific broth containing 100 μg/mL ampicillin, and this culture was allowed to incubate with shaking at 37 °C over the course of the day, whereupon in the late afternoon, it was used to innoculate an appropriate volume of terrific broth containing 100 μg/ml ampicillin. This large culture was allowed to incubate overnight at 37 °C with shaking. The procedure used for large scale plasmid isolation is based on the protocol presented in Sambrook, *et al.* (1989). The purified plasmid DNA was dissolved in an appropriate volume of TE buffer [pH 8.0]. Plasmid DNA preparations were quantified by acquiring the optical density of a given sample at 260 nm, where an $OD_{260} = 1.0$ corresponds to 50 μg of pure plasmid DNA. The OD_{280} of each sample was also taken, a ratio of $OD_{260}/OD_{280} = 1.7$ to 1.8 indicated a relatively pure plasmid preparation. Large scale plasmid preparations were stored at -20 °C until needed.

3.6 Cloning Strategies

The plasmid DNAs which were engineered during the course of this thesis work were constructed utilizing the protocols presented in section 3.5 of this document. Table 3.3 lists the names of the plasmids produced, their parent vector, cloning sites and insert origin.

Table 3.3 Overview of Plasmids Produced in this Thesis Work

Plasmid Produced	Parent Vector	Cloning Sites	Origin of Insert
19R-C/EBPα	pTZ-19R	*BamHI / PstI*	pCMV-C/EBPα
18R-LAP	pTZ-18R	*PstI*	pSCt-LAP
19R-PCK10	pTZ-19R	*EcoRI / BglII*	PCK10
19R-LAP	pTZ-19R	*Pst*I	pSCt-LAP
18R-GαN175	pTZ-18R	*EcoRI / BamHI*	pGαN175
MSV-GBF-F	MSV-C/EBPβ	*EcoRI / BamHI*	pRGX-GBF-F
MSV-C/EBPα anti	MSV-C/EBPβ	*EcoRI / BamHI*	pCMV-C/EBPα
MSV-C/EBPβ anti	MSV-C/EBPβ	*EcoRI / BamHI*	pSCt-LAP
MSV-GαN175	MSV-C/EBPβ	*EcoRI / BamHI*	pGαN175

The plasmids engineered during the course of this thesis work fall into two main groupings, those which were utilized as templates for *in vitro* transcribed ribonuclease protection analysis RNA probes and those which were expression vectors utilized in the production of stable rat hepatoma cell lines. The following paragraphs will briefly define the cloning strategies for each vector produced and reference the original citing for other vectors utilized during the construction.

The template utilized to produce a C/EBPα antisense RNA probe for ribonuclease protection analysis (19R-C/EBPα) was constructed by ligating the *BamHI / PstI* fragment

of pMSV-C/EBPα (Friedman, *et al.* 1989), corresponding to nucleotides +131 to +782 of the rat C/EBPα cDNA sequence, into *BamHI / PstI* digested pTZ-19R (United States Biochemical). This vector was linearized for probe production by digestion with *AvaI* which cuts at nucleotide +450 of the rat C/EBPα cDNA sequence, producing a 370 nucleotide RNA probe which protected 332 nucleotides of the C/EBPα mRNA.

The vector which was utilized as a template for ribonuclease protection analysis antisense RNA C/EBPβ probes (18R-LAP) was constructed by ligating the *PstI / PstI* fragment of pSCt-LAP (Descombes, *et al.* 1990), corresponding to nucleotides +246 to +736 of the rat C/EBPβ cDNA sequence, into the *PstI* site of pTZ-18R (United States Biochemical). Antisense orientation of insert containing clones was confirmed by digestion with *SmaI* at room temperature, where vectors containing insert in the proper orientation yielded an approximately 280 bp fragment. This vector was linearized for probe production by digestion with *HindIII* which cuts at nucleotide +310 of the multiple cloning site of pTZ-18R, producing a 545 nucleotide RNA probe which protected 490 nucleotides of the C/EBPβ mRNA.

The vector utilized as a template for the production of ribonuclease protection assay antisense RNA PEPCK probes (19R-PCK10) was constructed by ligating the *EcoRI / BglII* fragment of PCK10 (Yoo-Warren, *et al.* 1983), corresponding to nucleotides +70 to +300 of the rat PEPCK cDNA sequence into the *EcoRI / BamHI* sites of pTZ-19R. The vector was linearized for probe production by digestion with *XbaI* which cuts at position +285 of pTZ-19R, producing a 238 nucleotide probe which protected 230 nucleotides of the rat PEPCK-C mRNA.

The vector utilized as a template for ribonuclease protection analysis RNA probes for the detection of C/EBPβ antisense RNA (19R-LAP) was constructed by ligating the *PstI* / *PstI* fragment of pSCt-LAP into the *PstI* site of pTZ-19R. Sense orientation was confirmed by digestion of insert containing clones with *SmaI* at room temperature, where vector containing insert in the proper orientation would yield an approximately 180 bp fragment. This vector was linearized for probe production by digestion with *EcoRI* which cuts at nucleotide +315 of the multiple cloning site of pTZ-19R, producing a 550 nucleotide probe which protected 490 nucleotides of the C/EBPβ antisense RNA.

The vector utilized for the production of ribonuclease protection assay probes for detection of C/EBPα antisense RNA was produced by ligating the *EcoRI* / *BamHI* fragment of pGαN175 (Roesler, *et al.* 1998), corresponding to nucleotides +117 to +658 of the rat C/EBPα cDNA sequence, into the *EcoRI* / *BamHI* sites of pTZ-18R. This vector was linearized for probe production by digestion with *HindIII* which cuts at position +310 of the pTZ-18R multiple cloning site, producing a 573 nucleotide probe which protected 541 nucleotides of the C/EBPα antisense RNA.

The expression vector for GBF-F (MSV-GBF-F) utilized in the production of the MSV-GBF-F stable H4IIE cell line was constructed by ligating an *EcoRI* / *BamHI* fragment of pRGX-GBF-F (Olive, *et al.* 1996), corresponding to full length GBF-F, into the *EcoRI* / *BamHI* sites of MSV-C/EBPβ (Cao, *et al.* 1991), thus displacing the full-length C/EBPβ insert. The GBF-F *EcoRI* / *BamHI* fragment was derived from pRGX-GBF-F by PCR amplification utilizing a forward PCR primer designed to introduce an *EcoRI* site into the 5' un-translated region of the GBF-F cDNA sequence. The forward and reverse PCR

primers utilized to amplify the approximately 400 bp GBF-F *EcoRI* / *BamHI* fragment from pRGX-GBF-F where as follows:

GBF-F Forward: 5'-TATCGAATTCATGCCAGTGAAGGAT-3'

GBF-F Reverse: 5'-TATCGGATCCAAGCTTGCCGTC-3'

The expression vector used to express C/EBPα antisense RNA (MSV-C/EBPα anti) was constructed by sub-cloning the *PstI* / *BamHI* fragment of pMSV-C/EBPα, in antisense orientation, into the *PstI* / *BamHI* sites of the multiple cloning site of pBluescript® SK^+ (Stratagene) to produce pBluescript® SK^+-C/EBPα anti. The *EcoRI* / *BamHI* fragment of pBluescript® SK^+-C/EBPα anti was then ligated into the *EcoRI* / *BamHI* sites of MSV-C/EBPβ to produce MSV-C/EBPα anti.

The expression vector used to express C/EBPβ antisense RNA (MSV-C/EBPβ anti) was constructed by sub-cloning the *PstI* fragment of pSCt-LAP into the *PstI* site of pBluescript® SK^+ to produce pBluescript® SK^+-C/EBPβ anti. Vectors containing insert in the proper orientation were identified by digest with *SmaI* at room temperature, where positive clones would yield a 360 bp fragment. The *EcoRI* / *BamHI* fragment of pBluescript® SK^+-C/EBPβ anti was then ligated into the *EcoRI* / *BamHI* sites of MSV-C/EBPβ to produce MSV-C/EBPβ anti.

The expression vector used to express C/EBPα sense RNA (MSV-GαN175) was constructed by ligating the *EcoRI* / *BamHI* fragment of pGαN175 into the *EcoRI* / *BamHI* sites of MSV-C/EBPβ to produce MSV-C/EBPα sense. Vectors containing insert were

identified by *EcoRI / BamHI* diagnostic digest.

3.7 Isolation of Total RNA

Total RNA was isolated from whole liver tissue or from rat hepatoma H4IIE cells using TRIzol™ reagent. In the case of total RNA isolation from whole liver tissue, 100 mg of tissue was homogenized in 1 mL of TRIzol™ reagent. In the case of total RNA isolation from H4IIE cells, 400 μL of TRIzol™ reagent was added to each 100 mm plate and the cells were lysed by pipetting. In both situations, total RNA was isolated according to the manufacturers instructions. The total RNA pellet was dissolved in an appropriate volume of DEPC treated water. Total RNA samples were quantified by acquiring the optical density of a given sample at 260 nm, where an $OD_{260} = 1.0$ corresponds to 40 μg of pure total RNA. The OD_{280} of each sample was also taken, a ratio of $OD_{260}/OD_{280} = 1.6$ to 1.8 indicated a relatively pure total RNA sample. Total RNA samples were stored at -80 °C until use.

3.8 Synthesis of RNA Probes *in vitro*

RNA probes utilized in RNAse protection analysis (section 3.9) were synthesized *in vitro* by a protocol based upon that in Sambrook, *et al.* (1989). Briefly, the following were added, in order, into a screw-top vial at room temperature: 0.5 μg of the appropriate probe template (see section 3.6 for template cloning strategies), 7 mM DTT, 66 μg/mL

RNAse-free BSA, 18 units of RNA Guard ® placental RNAse inhibitor, 1 mM each of the three rNTPs other than rUTP, 1X transcription buffer containing, 40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine-HCl and 5 mM NaCl; 20 units of T7 RNA polymerase, 20 μM rUTP and approximately 50 μCi of [α^{32}P]-UTP. The components were mixed by a quick-spin in a microfuge and then incubated at 30 °C for 45 min to allow elongation of transcripts. After this incubation, 20 units of DNAse I was added to degrade the DNA template, and the reaction was allowed to incubate for 15 min at 37 °C, after which the tubes were placed on ice. Synthesized RNA probes were purified away from unincorporated nucleotide by gel exclusion chromatography on G-50 Sephadex™ spun-columns equilibrated with TEN buffer at 4 °C. TEN buffer contains 10 mM Tris-HCl [pH 8.0], 100 mM NaCl and 1 mM EDTA [pH 8.0]. *In vitro* transcribed RNA probes were stored at -80 °C until used.

3.9 Ribonuclease Protection Analysis

Ribonuclease (RNAse) protection analysis was generally carried out utilizing 10 μg of total RNA sample and approximately 350000 cpm of labelled RNA probe synthesized as outlined in section 3.8. Total RNA and labelled probe were placed in a 1.5 mL microfuge tube, 5 units of RNAse free RQI DNAse was added and was allowed to incubate at 37 °C for 20 min. After DNAse treatment, the RNA was dried down in a vacuum centrifuge. The dried RNA pellet was solubilized in 20 μL of RNAse protection hybridization buffer containing 80% formamide, 40 mM PIPES [pH 6.4], 1 mM EDTA

[pH 8.0] and 400 mM NaCl. It should be noted that all formamide used for RNA work was de-ionized by mixing with AG® 501-X8 mixed bed resin and was then filtered. The RNAse protection samples were then denatured by boiling for 4 min and then allowed to hybridize at 42 °C overnight. The next morning, the RNAse protection samples were digested by the addition of 350 µL of RNAse digestion buffer containing 10 mM Tris-HCl [pH 7.4], 250 mM NaCl, 5 mM EDTA [pH 8.0], 11 µg/mL RNAse A and 4 units of RNAse T_1, followed by incubation at 30 °C for 20 min. The RNAse digested samples were then treated with 0.5% SDS and 65 µg/mL proteinase K at 37 °C for 20 min. The samples were then extracted with 400 µL of phenol : chloroform (1:1), where after 350 µL of the aqueous phase was removed to a fresh 1.5 mL microfuge tube. The protected total RNA - RNA probe hybrids were precipitated with the addition of 10 µg of yeast total RNA and 1 mL of absolute ethanol and incubation at -20 °C for 30 min. The precipitated RNA hybrids were pelleted by centrifugation at 12 000 rpm at 4 °C for 15 min. The radioactive supernatant was removed and the pellet washed in DEPC treated 70% ethanol, and the pellet allowed to dry at 37 °C for 15 min. After drying, the RNA hybrid pellet was dissolved in 20 µL of denaturing polyacrylamide gel sample buffer (see section 3.5.6) which involved alternate vigorous vortexing and incubation at 37 °C for 5 min periods. After solubilization of the labelled RNA hybrids in the sample buffer, the samples were denatured by boiling for 4 min and then plunged on ice. The denaturing 6% polyacrylamide 8 M urea gels used for resolving RNAse protection analysis were identical in composition to the sequencing gels outlined in section 3.5.6, and the running buffer for electrophoresis was also 0.5X TBE. After loading of the samples onto a Bio-rad Protean®

II xi cell, electrophoresis was allowed to proceed at 120 volts until sufficient resolution of the samples had been obtained. Following electrophoresis, the denaturing polyacrylamide gels were dried on a vacuum gel drier overnight and then subjected to autoradiography using Kodak X-OMAT film at -80 °C. Autoradiograms were quantified by densitometry utilizing the Un-Scan-It™ automated digitizing system (Silk Scientific Inc.).

3.10 Preparation of Random-prime Labelled cDNA Probes

The DNA fragments utilized for cDNA probe synthesis are listed in Table 3.4. DNA fragments used as cDNA probe templates were excised from their parent plasmids by appropriate restriction enzyme digests and then gel isolated (section 3.5.3) and dissolved in sterile water at a concentration of approximately 25 ng/μL. The DNA fragments were first denatured by boiling for 10 min and were then plunged on ice. Probe synthesis was begun by adding the following into a screw-top vial on ice: 100 ng of the appropriate DNA fragment, 75 μM dNTP mixture, 1X hexanucleotide mixture, approximately 50 μCi of [α-^{32}P] dCTP, 2 units of labelling grade Klenow fragment of DNA polymerase I and sterile water for a total volume of 20 μL. The hexanucleotide mixture contains, at 1X concentration, 50 mM Tris-HCl [pH 7.2], 10 mM $MgCl_2$, 100 μM DTE, 200 μg/mL BSA and hexanucleotides at a concentration of 6.25 A_{260} units/mL. Random prime probe synthesis was allowed to occur for 1 h at 37 °C. Random cDNA probes were purified away from unincorporated nucleotide by gel exclusion chromatography utilizing G-50 Sephadex™ spun-columns equilibrated with TEN at 4 °C.

Table 3.4 Templates Utilized for Random Prime cDNA Probe Synthesis

cDNA Probe	Restriction Fragment	Parent Vector	Vector Source
C/EBPα	*EcoRI / BamHI*	pGαN175	W.J. Roesler Laboratory, University of Saskatchewan, SK Canada
GBF-F	*EcoRI / BamHI*	pRGX-GBF-F	C.R. Vinson Laboratory, NIH, Maryland, USA
18S-RIBO	*HindIII*	pRIBO	R.V. Guntaka Laboratory, Columbia University, New York, USA

References for pGαN175 and pRGX-GBF-F can be found in section 3.6 of this document. The parent vector pRIBO was first described by Katz, *et al.* (1983).

3.11 Northern Blotting

Total RNA sample preparation for Northern blotting was conducted as follows: 20 μg of a given total RNA sample was incubated for 10 min at 65 °C in RNA sample buffer containing 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate [pH 7.0] and 1 mM EDTA [pH 8.0]), 50% formamide and 16% formaldehyde. Following this incubation samples were placed on ice and 2.5 μL of RNA sample loading buffer was added

containing 50% glycerol and traces of bromophenol blue. Total RNA samples were electrophoresized on 1% agarose / 16% formaldehyde gels which were prepared as follows: one gram of agarose was dissolved in 10 mL of 10X MOPS buffer and 74 mL of DEPC treated water and heated to approximately 80 °C to dissolve the agarose. After sufficient cooling 16 mL of formaldehyde was added and the gel poured into a horizontal gel cast with an appropriate well form. Electrophoresis was conducted utilizing 1X MOPS as a running buffer, RNA samples were loaded and the gel run at 30 volts until an appropriate amount of resolution had been achieved. After electrophoresis, the gel was washed in two or three changes of DEPC treated water to remove the excess formaldehyde. After these washes the gel was soaked in 0.05 N NaOH for 15 min to partially hydrolyse the RNA and to facilitate complete transfer to the charged membrane upon capillary elusion. After the NaOH soak, the gel was then soaked in 20X SSC for 45 min at room temperature (20X SSC contains 3 M NaCl and 300 mM sodium citrate). The setup for capillary elution was essentially as described in Sambrook, *et al.* (1989). Following capillary transfer the RNA was fixed to the charged membrane by placing the membrane, RNA side up, on three pieces of filter paper soaked in 0.05 N NaOH for 20 min at room temperature. Following this fix the membrane was washed in 6X SSC for 5 min at room temperature. The membrane was then pre-hybridized at 65 °C for at least 2 h in a solution containing 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS, 10% dextran sulphate and 100 μg/mL denatured salmon sperm DNA. Denhardt's solution was made up as a 50X stock containing 1% (w/v) BSA, 1% (w/v) Ficoll 400 and 1% (w/v) PVP; SSPE was made up as a 20X stock containing 3.6 M NaCl, 200 mM sodium phosphate and 20 mM EDTA [pH

8.0]. After pre-hybridization, an appropriate amount of labelled cDNA probe (section 3.10) was denatured by boiling for 5 min and then added to the pre-hybridization solution. The membrane in pre-hybridization - cDNA probe solution was then allowed to hybridize overnight at 65 °C. Following hybridization, the membrane was washed two times in 2X SSPE, 0.1% SDS for 20 min at room temperature followed by a single wash in 1X SPPE, 0.1% SDS for 15 min at 65 °C. The wet membrane was heat-sealed in plastic to prevent it from drying and subjected to autoradiography utilizing Kodak X-OMAT film at -80 °C.

3.12 RNA Slot Blotting

Prior to application of RNA samples to the charged membrane by slot blotting, the membrane itself was wetted in 6X SSC buffer and assembled in a Bio-Rad Bio-Dot® SF slot-blot manifold. Each of the manifold's slots were pre-washed with 500 μL of 6X SSC. Total RNA samples were combined with three volumes of RNA slot-blot loading buffer containing 50% formamide, 16% formaldehyde and 1X MOPS buffer and were then incubated at 65 °C for 5 min. After incubation, samples were placed on ice and one volume of ice-cold 20X SSC was added. Each of the total RNA samples was then applied to an individual slot of the manifold and washed through with 500 μL of 6X SSC. After application of the total RNA samples, the manifold was disassembled and the charged membrane was fixed with alkali by placing it RNA side up upon several layers of filter paper soaked in 0.05 N NaOH for 5 min at room temperature. Following alkali fixation, the membrane was briefly washed in 2X SSC. The membrane was then pre-hybridized for

2 h at 42 °C in a solution containing 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS, 10% dextran sulphate, 50% formamide and 20 μg/mL denatured salmon sperm DNA. After pre-hybridization denatured 18S-RIBO cDNA probe was added at approximately 1 x 10^6 cpm/mL and allowed to hybridize overnight at 42 °C. After hybridization, the membrane was washed, initially in two washes of 2X SSPE, 0.1% SDS for 10 min at room temperature, followed by one wash in 1X SPPE, 0.1% SDS for 15 min at 65 °C and a final wash in 0.1X SSPE, 0.1% SDS for 10 min at 65 °C. The membrane was then heat sealed in plastic and subjected to autoradiography utilizing Kodak X-OMAT film at -80 °C.

3.13 Harvesting of Protein Lysates

After appropriate experimental manipulations (see section 4), adherent cells or whole rat liver were washed in several mL of ice-cold 1X phosphate-buffered saline (PBS). Phosphate-buffered saline contains 140 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4 - 7 H_2O and 18 mM KH_2PO_4, all buffered to a pH of 7.4. Whole rat liver was homogenized in a suitable volume of ice-cold RIPA buffer and then incubated on ice for 30 min. After washing, adherent cells were scraped from the plates into 200 μL of RIPA buffer and placed into pre-chilled 1.5 mL microfuge tubes and then incubated on ice for 30 min. RIPA buffer contains 1X PBS, 1% Nonidet NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, 100 μg/mL PMSF, 2 μg/mL aprotinin, 1 mM sodium orthovanadate and 1 μg/mL pepstatin A. Following incubation on ice, lysate samples were sonicated for 5 sec at a 75% setting. Lysate samples were then centrifuged at 12 000 rpm for 15 min at 4 °C to pellet

cellular debris. Following centrifugation, the supernatant was removed to a fresh tube and this lysate was stored at -80 °C until use. An aliquot of the lysate was first removed however, and an appropriate volume of 5X protein lysate sample buffer was added and the sample boiled for 5 min whereupon it was analysed by SDS-PAGE. Protein lysate sample buffer contains 50 mM Tris-HCl [pH 6.8], 2% SDS, 400 mM 2-mercaptoethanol, 10% glycerol and traces of bromophenol blue. Protein lysates were quantified using Bio-Rad Protein Assay Dye reagent in a protocol based upon that first described by Bradford, *et al.* (1976).

3.14 Electrophoresis of Protein Lysates on SDS-PAGE

Protein lysates were generally resolved on 10 % SDS-PAGE gels which contained 10 % acrylamide : N,N'-methylene-bis-acrylamide (29.2 : 0.8), 375 mM Tris-HCl [pH 8.8] and 0.1% SDS in the resolving gel, and 5% acrylamide : N,N'-methylene-bis-acrylamide (29.2:0.8), 130 mM Tris-HCl [pH 6.8] and 0.1% SDS in the stacking gel. Polymerization was initiated by the addition of 0.1% APS and 0.04% TEMED. The running buffer utilized for SDS-PAGE contained 25 mM Tris-HCl, 250 mM glycine [pH 8.3] and 0.1% SDS. In order to partially confirm that protein lysates had been appropriately quantified, an equivalent amount of each lysate, generally 20 μg, was run out on 10% SDS-PAGE gels loaded into Bio-Rad Mini Protean® II apparatus, at 120 volts, constant voltage until the bromophenol blue dye marker had just run off the gel. The gels were then stained in Coomassie® Brilliant Blue stain for 20 min and then destained in a buffer containing 30%

methanol and 10% acetic acid. An equivalent amount of staining in each lane indicated relatively good quantification and such lysates where then deemed suitable for Western analysis.

3.15 Western Blotting

Quantified protein lysates in SDS-PAGE sample buffer, generally 20 μg, were loaded onto 10% SDS-PAGE gels with appropriate protein molecular weight standards and resolved at 90 volts, constant voltage, until the bromophenol blue tracker dye had passed through the stacking gel. The voltage was then increased to 120 volts, constant voltage and electrophoresis was allowed to proceed until the tracker dye had just run off the gel. Resolved protein lysates were then transferred to nitrocellulose membranes utilizing a Bio-Rad Trans-blot apparatus, operated at 120 constant volts for 45 min at 4 °C. The transfer buffer used in the trans-blot contained 39 mM glycine, 48 mM Tris-HCl and 20% methanol. After transfer, the nitrocellulose membranes were briefly washed in PBST buffer containing 1X PBS and 0.04% Tween-20. Nitrocellulose membranes were then blocked in PBST buffer containing 5% Bio-Rad Blotting Grade Blocker Non-Fat Dry-Milk for 2 h at room temperature. Following blocking the membranes were placed in fresh PBST buffer containing 5% blocking reagent as well as a 1:1000 dilution of the appropriate rabbit primary antibody. The membranes and antibody were then allowed to hybridize with gentle shaking at 4 °C overnight. After overnight hybridization, the membranes were washed three times for 5 min each in fresh PBST buffer containing 5% Bio-Rad blocking

reagent. A 1:10 000 dilution of goat anti-rabbit secondary antibody conjugated to horse radish peroxidise was then added and allowed to hybridize with the membrane for 45 min at room temperature. Following this final hybridization, the membranes were then again washed three times for 5 min each in PBST buffer containing 5% Bio-Rad blocking reagent, followed by several brief washes in straight PBST buffer. Antibody-protein complexes were then visualized utilizing the NEN-Mandel Renaissance® chemiluminescent detection kit and Kodak X-OMAT film at room temperature. Autoradiograms were quantified by densitometry utilizing the Un-Scan-It™ automated digitizing system (Silk Scientific Inc.).

3.16 Preparation of Stable Transfected Rat Hepatoma H4IIE Cells

The protocols for production of expression vectors utilized in the production of stable cell lines have been outlined in section 3.6. Prior to the day of transfection wild-type rat hepatoma H4IIE cells were passaged with a 1:4 dilution so as to render them at approximately 50-70% confluence the next morning. The next day the cells were transfected with 15 μg total DNA per plate. Cells were transfected with an appropriate expression vector and a vector containing a selectable marker for gentamicin (G418) resistance, pSV_2-neo (InforMax, Inc.). The molar ratio of selectable marker vector to expression vector utilized was approximately 1:10. The transfection mixture was composed of two separate solutions which were combined slowly, dropwise, and then incubated for 30 min at room temperature to allow the formation of a fine $CaCl_2$-DNA

precipitate. The first solution contained appropriate amounts of expression vector and pSV_2-neo in sterile water and contained 250 mM $CaCl_2$ (125 mM final concentration in complete transfection mix). The second solution contained 50 mM HEPES [pH 7.2] (25 mM final), 250 mM NaCl (125 mM final) and 1.5 mM $NaPO_4$ [pH 7.0] (750 μM final). After formation of the transfection precipitate the cell media was aspirated and plates were washed with PBS. The transfection mix was then applied in a volume of 1 mL per plate and allowed to remain in direct contact with the cells for 20 min at room temperature. Following this incubation, 9 mL of cell culture media was added to the plates and they were then allowed to incubate for 6 h at 37 °C and 5% CO_2. Following this incubation the cells were glycerol-shocked by applying 2 mL per plate of a solution containing 25 mM HEPES [pH 7.2], 125 mM NaCl and 15% glycerol. This solution was allowed contact with the cells for 2.5 min at 37 °C, where upon it was diluted out by addition of large volumes of cell culture media which was promptly aspirated. The plates were then washed two or three times with PBS and then 10 mL of fresh cell culture media was added to each plate. The transfected cells were allowed to incubate for at least 48 h at 37 °C and 5% CO_2 whereupon, if they were confluent, they were passaged with a 1:4 dilution into cell culture media containing 400 μg/mL gentamicin (G418). Selection was allowed to proceed until individual clones had reached a diameter of 3-4 mm whereupon they were isolated in separate 24-well plate wells in media containing 100 μg/mL gentamicin (G418). As clones reached confluence, they were transferred up to increasingly larger diameter wells until they could be passaged with dilution in 100 mm plates, and at this point liquid nitrogen

DMSO cell stocks were prepared and expression analysis could begin. All stable cell lines were cultured indefinitely in 100 μg/mL gentamicin (G418) to maintain selection pressure.

4. RESULTS

4.1 Hormonal Regulation Studies

4.1.1 Rationale for Use of the Rat Hepatoma H4IIE Cell Line

The rat hepatoma H4IIE cell line was chosen for studies examining the hormonal regulation of liver expressed C/EBP isoforms because of its known sensitivity to the hormones of interest and its documented similarity to the differentiated hepatocyte (Pitot, *et al.* 1964). In addition, much of the work done to define the hormonal sensitivity of the PEPCK-C gene was performed in this cell line (Sasaki, *et al.* 1984; Faber, *et al.* 1993 and O'Brien, *et al.* 1994), which provided a convenient internal control to ensure that an applied hormone was having a measurable effect on gene expression within the cell.

4.1.2 Effect of Hormones on mRNA Accumulation of C/EBP Isoforms in Rat Hepatoma H4IIE Cells

The first experiment conducted was designed to confirm that the H4IIE cell line was indeed responsive to the hormones which were to be utilized within the study. Prior to treatment with hormones, cells were serum-starved by aspirating off the serum-containing DMEM cell medium, washed with PBS, and refed with fresh DMEM medium

lacking serum and then incubated for 24 hours. The cells were then treated with hormones for 4 hours, either as individual treatments or in various combinations with other hormones. After the treatment total RNA was isolated from the cells and the changes in PEPCK-C mRNA were determined by ribonuclease protection analysis (Figure 4.1 shows a representative autoradiogram). The result of this analysis revealed that H4IIE cells were indeed a suitable model system to study changes in gene expression induced by hormonal signals, as the effects of hormones upon PEPCK-C mRNA levels within these cells were as expected (see section 2.2.2). The hormones utilized in these studies included insulin, the synthetic glucocorticoid dexamethasone (Arth, *et al.* 1958) and glucagon, whose effects were induced indirectly by application of the cAMP analogue 8-chlorophenylthio - cyclic adenosine monophosphate (8-CPT-cAMP). The use of 8-CPT-cAMP and other cAMP analogues to mimic the effects of glucagon has been described previously in the literature (Sasaki, *et al.* 1984 and Park, *et al.* 1993). These analogues are of particular value in cell culture-based systems in that they can pass directly through the cell membrane and thus do not necessarily require the expression of the glucagon receptor by the cell, as well as having been proven to be resistant to the effects of cAMP phosphodiesterases (Beebe, *et al.* 1985).

Having confirmed the hormonal responsiveness of the H4IIE cells, the characterization of the patterns of C/EBPα and C/EBPβ hormonal regulation were undertaken. H4IIE cells were serum-starved overnight and then treated for 4 and 8 hours with various hormones, independently or in combination. Total RNA was then isolated from the cells and subjected to ribonuclease protection analysis.

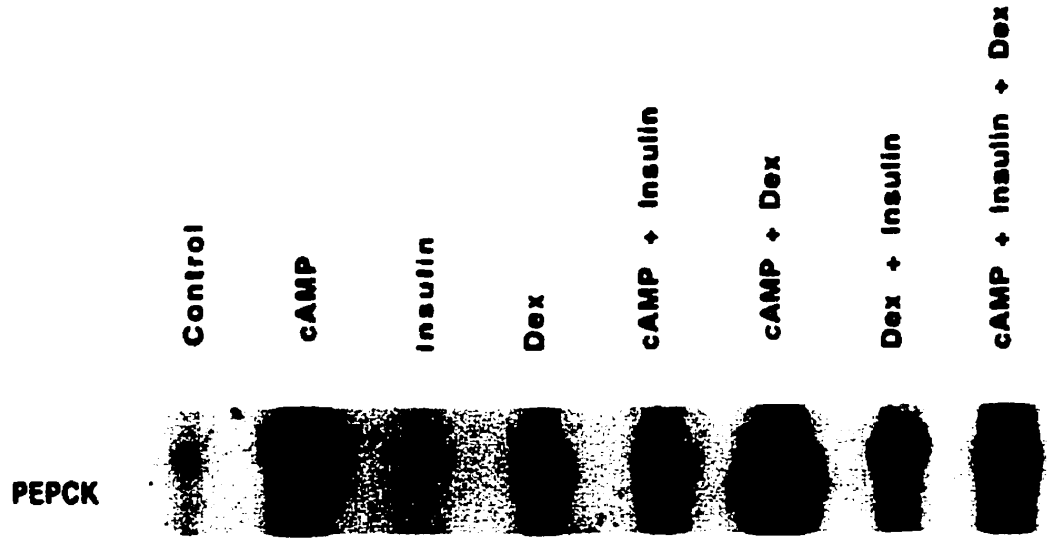

Figure 4.1 The effect of various hormonal treatments on PEPCK-C mRNA accumulation in H4IIE cells. Changes in PEPCK-C mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. The hormones with which H4IIE cells were treated included 200 μM 8-CPT-cAMP (cAMP), 5 nM insulin and 1 μM dexamethasone (Dex). All hormonal treatments were for a 4 hour time period. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown).

Figure 4.2a shows representative autoradiograms and the averages of four independent experiments and Figure 4.2b displays the means ± S.E. of four independent experiments in a graphical format; revealing the changes in C/EBPα and C/EBPβ mRNA levels relative to controls after treatment with various hormones.

One of the most outstanding observations arising from these experiments was the robust induction in both C/EBPα and C/EBPβ mRNA accumulation as a result of dexamethasone treatment of rat H4IIE cells either alone or in combination with other hormones. The effects of dexamethasone on C/EBPα mRNA were most significant at the 4 hour time point where levels were increased 3-fold on average over controls. This inductive effect was diminished to a 2-fold increase over control by the 8 hour time point. The effect of dexamethasone on C/EBPβ mRNA accumulation was greater than that on C/EBPα, increasing C/EBPβ mRNA 4-fold over control at the 4 hour time point and increasing to 8.5-fold over controls on average by 8 hours. Furthermore, when dexamethasone was applied to H4IIE cells in various combinations with the other hormones, its effects upon C/EBPα and C/EBPβ mRNA levels were generally additive with the effects of the other hormones. Thus it would appear that glucocorticoids are a dominant activator of C/EBP expression within the rat hepatoma cell.

The effects of 8-CPT-cAMP upon C/EBPα mRNA accumulation were modest at best, with the largest induction being 2-fold on average at 8 hours. The effects of 8-CPT-cAMP on C/EBPβ mRNA accumulation were greater than its effects on C/EBPα mRNA, with a average 2-fold induction of C/EBPβ message levels observed at the 4 hour time point which increased to 4.5-fold by 8 hours. When 8-CPT-cAMP was applied to H4IIE

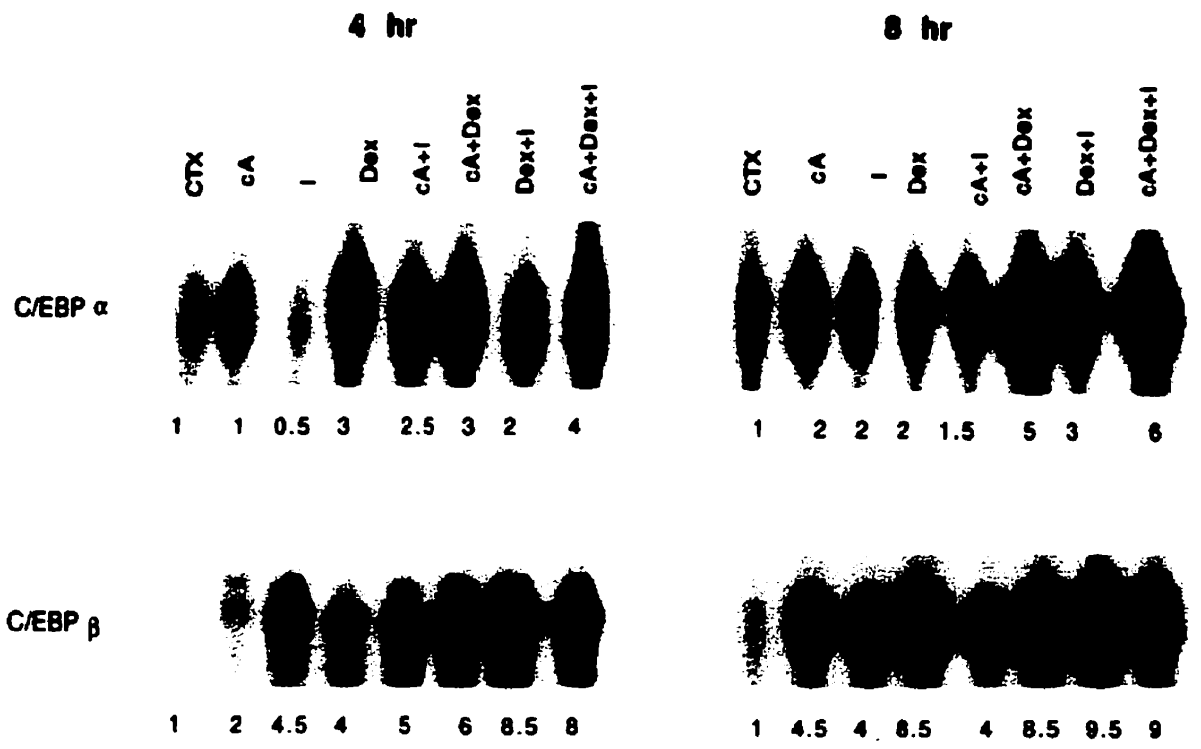

Figure 4.2a The effects of various hormonal treatments on C/EBPα and C/EBPβ mRNA accumulation in H4IIE cells. Changes in mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. H4IIE cells were treated with either 200 μM 8-CPT-cAMP (cA), 5 nM insulin (I), 1 μM dexamethasone (Dex) or various combinations thereof for 4 and 8 hours; controls are abbreviated as CTX. Each panel shows a representative autoradiogram, the values below are the relative mRNA level (where controls were assigned a value of 1) averaged from four independent experiments. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown).

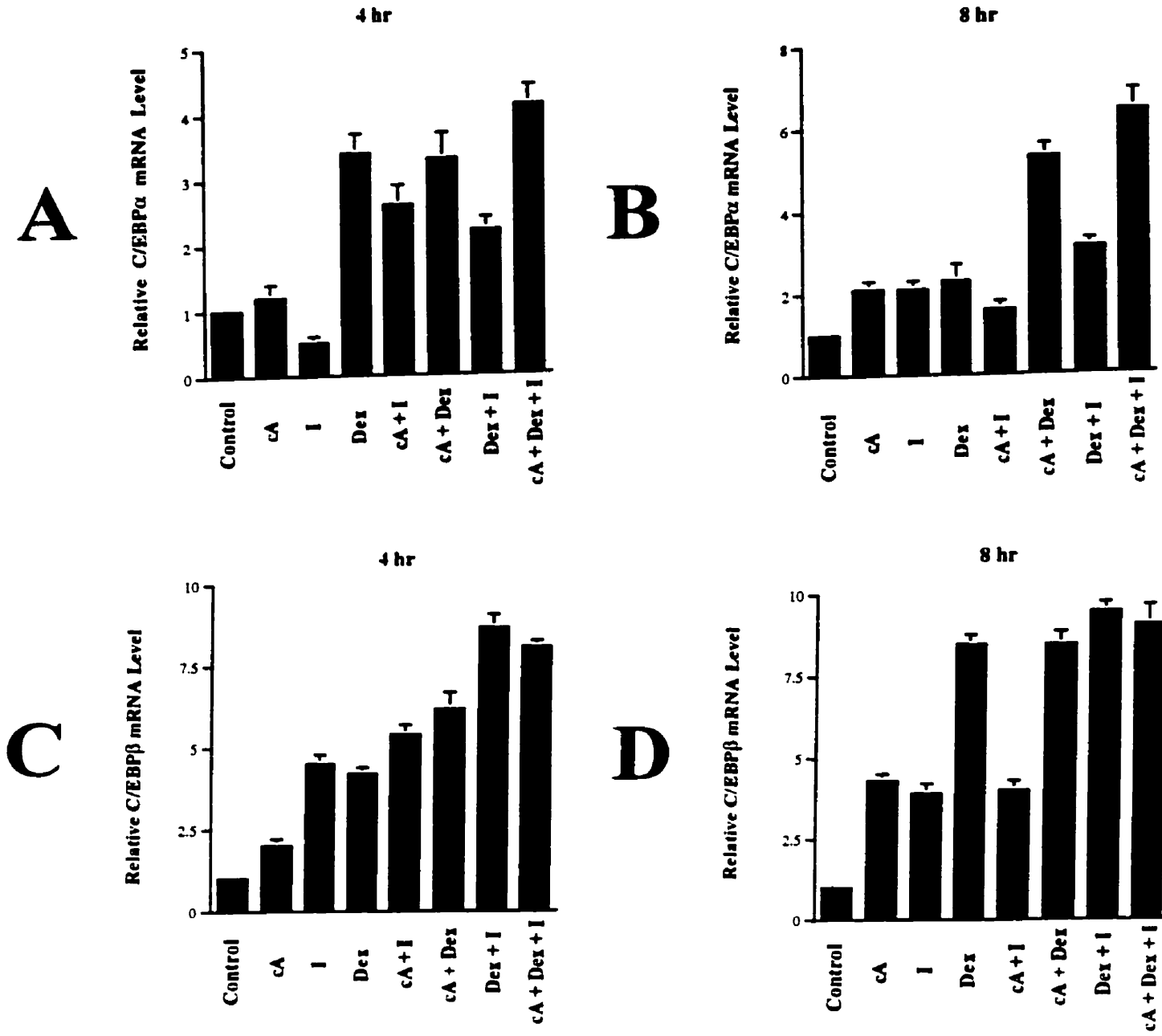

Figure 4.2b Graphical representation of the effects of various hormonal treatments on C/EBPα (Panels A and B) and C/EBPβ (Panels C and D) mRNA accumulation in H4IIE cells. Changes in mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. H4IIE cells were treated with either 200 μM 8CPT-cAMP (cA), 5 nM insulin (I), 1 mM dexamethasone (Dex) or various combinations thereof for 4 and 8 hours. The levels of C/EBPα or C/EBPβ mRNA in untreated cells were arbitrarily assigned a value of 1. The above graphs displays the means ± S.E. of four independent experiments.

cells in combination with dexamethasone, the effects on C/EBPα and C/EBPβ message were generally additive. However, when 8-CPT-cAMP was applied to H4IIE cells in combination with insulin there were no additive effects upon C/EBP isoform expression. An interesting exception is the effect of cAMP and insulin in combination upon C/EBPα mRNA in H4IIE cells at the 4 hour time point, where a 2.5-fold on average induction was observed, although 8-CPT-cAMP generally had no effect on C/EBPα expression at this time point and insulin alone was seen to be inhibitory.

The effect of insulin upon C/EBPα message accumulation appeared to be bi-phasic, in that at the 4 hour time point insulin appeared to inhibit C/EBPα expression by 50%, whereas by the 8 hour time point a 2-fold induction of C/EBPα mRNA over control was observed. Insulin inhibited the inductive effect of dexamethasone on C/EBPα mRNA levels at the 4 hour time point, but was roughly additive with the inductive effect of dexamethasone on C/EBPα expression by 8 hours. Unlike the effects of insulin upon C/EBPα expression within H4IIE cells, insulin induced C/EBPβ expression (approximately 4-fold) at both time points measured.

In order to insure that equivalent amounts of RNA were utilized for each sample in all RNA based protocols, 5 μg from each experimental sample was subjected to RNA slot-blot analysis using an 18S ribosomal RNA cDNA probe, where equivalent 18S rRNA signals were considered to be indicative of acceptable RNA sample equilibration. Figure 4.3 shows a representative autoradiogram of an 18S rRNA slot-blot prepared using the RNA samples utilized in the ribonuclease protection analysis shown in Figure 4.2.

Figure 4.3 A representative autoradiogram of a 18S ribosomal RNA slot-blot of the total RNA samples utilized in the ribonuclease protection analysis shown in Figure 4.2. Total RNA samples isolated as outlined in section 3.7 were subjected to RNA slot-blot analysis as outlined in section 3.12. Total RNA samples were considered to be of equivalent amounts if the 18S rRNA signal of an individual sample did not vary by more than 10% from the other samples. The 18S rRNA signal could be quantified either by densitometry or by liquid scintillation counting of the individual "slots" of the charged membrane.

It should also be noted that, in order to insure that the changes in C/EBP isoform gene expression observed were specific, the mRNA levels of the cAMP response element binding protein (CREB) were also assessed (data not shown), and were observed to be unaltered in comparison to controls in all treated samples. This finding was in agreement with previously published work which had demonstrated that CREB mRNA levels are not hormonally regulated in rat liver or rat liver-derived cells (Crosson, *et al.* 1996).

4.1.3 Effect of Hormones on the Protein Levels of C/EBPα and C/EBPβ in Rat Hepatoma H4IIE Cells

The next area of experimentation involved determining whether the effects of various hormones on C/EBPα and C/EBPβ expression at the level of mRNA accumulation corresponded to changes observed in the steady-state levels of C/EBP isoform proteins. Western analysis was performed on protein lysates prepared from hormone-treated H4IIE cells at 8 and 16 hour time points. The time points used in this study were greater than those used for the analysis of mRNA accumulation in order to allow C/EBP isoform proteins to accumulate to a steady-state level following gene transcription. Figure 4.4a is a representative autoradiogram of four independent experiments, while Figure 4.4b displays the means ± S.E. of four independent experiments in graphical format. The protein levels of the RNA polymerase II general transcription factor TFIIEα were also assessed in each lysate in order to insure an equivalent amount of total protein had been utilized for each sample subjected to Western analysis. In general, the changes in C/EBPα

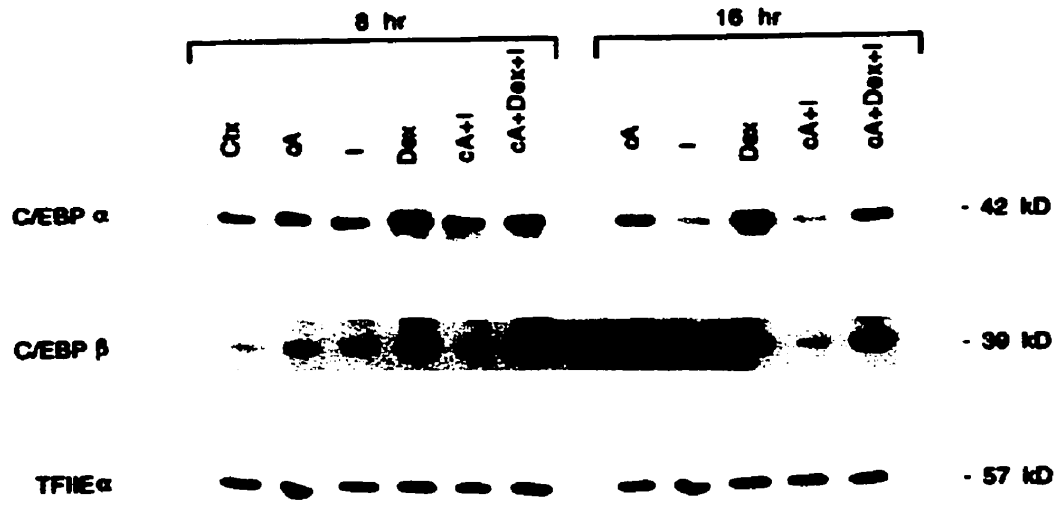

Figure 4.4a The effect of various hormonal treatments on C/EBPα and C/EBPβ protein levels in H4IIE cells. Changes in protein accumulation were assessed by Western analysis as outlined in section 3.15. H4IIE cells were treated with either 1 mM 8-CPT-cAMP (cA), 5 nM insulin (I), 1 μM dexamethasone (Dex) or various combinations thereof for 8 and 16 hours. Controls are abbreviated as Ctx. Each panel shows a representative autoradiogram of four independent experiments. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα.

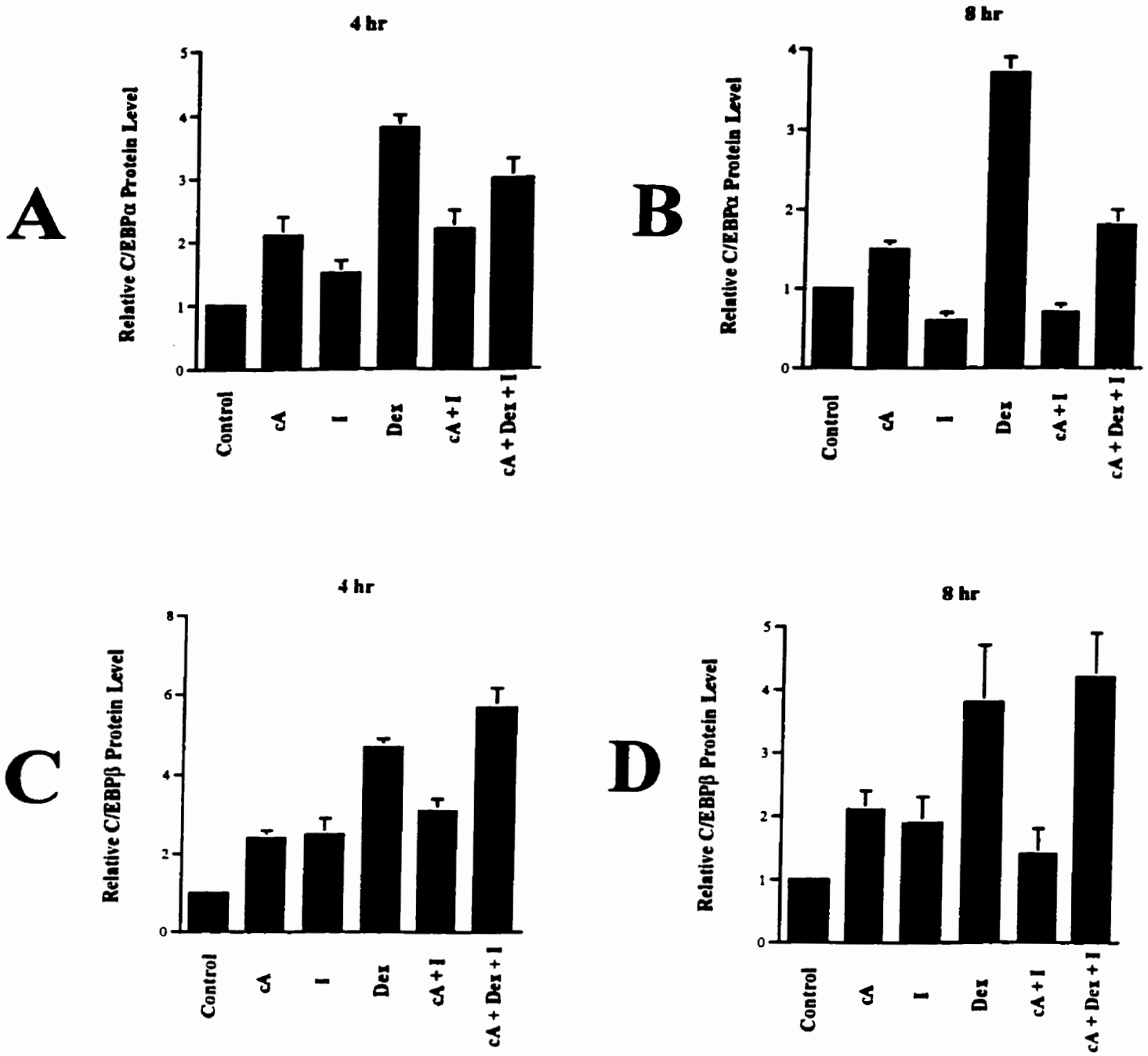

Figure 4.4b Graphical representation of the effects of various hormonal treatments on C/EBPα (Panels A and B) and C/EBPβ (Panels C and D) protein levels in H4IIE cells. Changes in protein accumulation were assessed by Western analysis as outlined in section 3.15. H4IIE cells were treated with either 1 mM 8CPT-cAMP (cA), 5 nM insulin (I), 1 mM dexamethasone (Dex) or various combinations thereof for 8 and 16 hours. The levels of C/EBPα or C/EBPβ protein in untreated cells were arbitrarily assigned a value of 1. The above graphs displays the means ± S.E. of four independent experiments.

and C/EBPβ protein levels directly parallelled the changes observed in mRNA accumulation. The most notable effects upon C/EBP protein levels were caused by dexamethasone, which parallelled the changes observed in mRNA accumulation. Treatment with cAMP had a slight inductive effect upon C/EBPα protein levels at both time points. The induction of C/EBPβ protein levels by cAMP appeared to be more significant than that of C/EBPα, but was more acute in that the inductive effect was only sustained up to the 8 hour time point. Insulin also induced C/EBPβ protein levels up to the 8 hour time point. The inhibitory effect of insulin upon C/EBPα mRNA accumulation was also observed at the level of its protein, however the effect appeared to be delayed in that it did not appear until the 16 hour time point. The changes in C/EBPα and C/EBPβ protein levels as a result of combination hormonal treatment were generally not additive, and furthermore insulin was seen to inhibit the effects of both cAMP and dexamethasone on C/EBPα protein levels at both the 8 and 16 hour time points, much like what was observed with changes in C/EBPα mRNA accumulation at the 8 hour time point.

4.1.4 Effect of cAMP Bolus on C/EBP Isoform mRNA Levels in Rat Liver

The aforementioned studies regarding the effect of cAMP upon C/EBP isoform mRNA accumulation and protein levels had suggested that the β-isoform of C/EBP was more responsive to this second messenger than was the α-isoform in a hepatoma cell line. We also wished to determine whether C/EBP isoforms were regulated *in vivo* by cAMP

within the intact rat liver. As outlined in section 3.4, male Sprague-Dawley rats were subjected to a theophylline / 8-CPT-cAMP bolus, total RNA was isolated from their livers, and the effect of the cAMP bolus upon C/EBP isoform mRNA accumulation was assessed by ribonuclease protection analysis. Figure 4.5 shows the results of this 8-CPT-cAMP bolus experiment. No significant difference was observed in hepatic C/EBPα mRNA levels between control and 8-CPT-cAMP treated rats. This observation correlates with the relatively minor effects of 8-CPT-cAMP upon C/EBPα mRNA accumulation within the rat hepatoma H4IIE cell line (section 4.1.2). The levels of C/EBPβ mRNA however, were elevated nearly 7-fold over controls in the livers of the same treated animals, indicating that the *in cyto* observation that C/EBPβ is the major 8-CPT-cAMP regulated hepatic C/EBP isoform can be extended into the *in vivo* setting. In order to assess whether changes in C/EBP isoform expression were unique, the levels of PEPCK-C and CREB mRNA were also measured (data not shown). The levels of PEPCK-C mRNA were considerably elevated over controls in the livers of cAMP bolus treated rats and the levels of CREB mRNA were unchanged, as would be expected based upon previous findings (section 2.2.2 and Crosson, *et al.* 1996, respectively).

4.1.5 Effect of Streptozotocin-Diabetes on C/EBP Isoform Expression in Rat Liver

Given that the results presented in section 4.1.2 thru 4.1.4 demonstrated that hepatic C/EBP isoforms are regulated by glucose homeostasis-affecting hormones, and because of the acknowledgment that C/EBP isoforms are important regulators of energy homeostasis

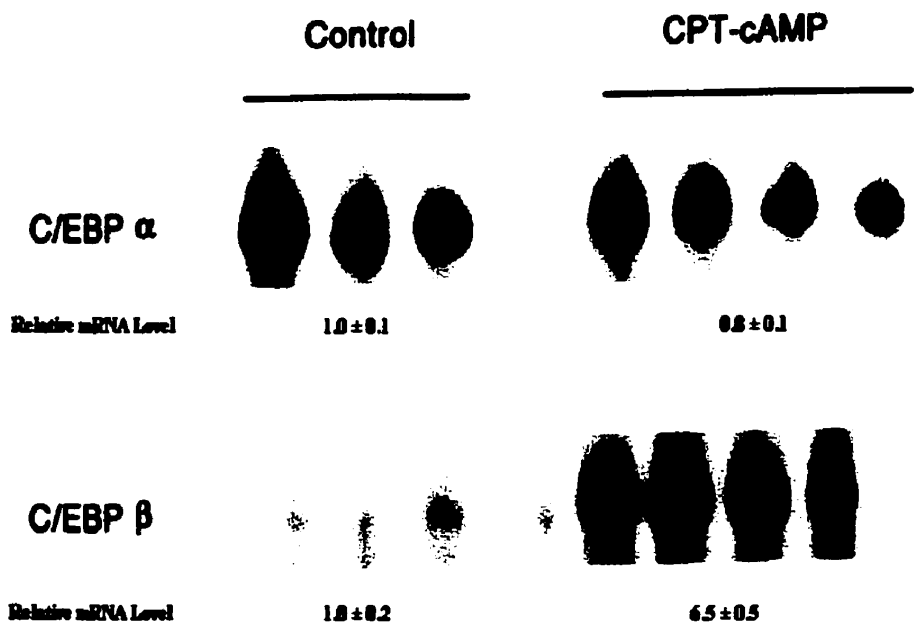

Figure 4.5 Effect of 8-CPT-cAMP on the mRNA levels of C/EBPα and C/EBPβ in rat liver. Male Sprague-Dawley rats were given intraperitonial injections of 8-CPT-cAMP/theophylline (CPT-cAMP, n=4) or saline carrier (Controls, n=3) as outlined in section 3.4, total RNA was then isolated from the livers (section 3.7) and analysed for the levels of C/EBPα and C/EBPβ mRNA by ribonuclease protection analysis (section 3.9). The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown). Values shown are the means ± S.E..

(section 2.3.3), we next examined the effects of streptozotocin-induced diabetes on hepatic C/EBP isoform expression. The effects of diabetes and subsequent insulin treatment upon mRNA levels of C/EBPα and C/EBPβ in rat liver are shown in Figure 4.6. The levels of C/EBPβ mRNA were not altered in streptozotocin-diabetic rat livers and subsequent insulin treatment had no further effect. However, the levels of C/EBPα mRNA in diabetic rat liver were reduced by 60-80% relative to controls as assessed by densitometry.

The levels of C/EBPα and C/EBPβ proteins were also assessed in these same livers by Western analysis (Figure 4.7). No change was seen in either of the translation forms of C/EBPβ (section 2.3.1.3) in streptozotocin-diabetic or diabetic-insulin treated rat livers relative to controls. The levels of C/EBPα protein were decreased in diabetic and insulin-treated rat livers, parallelling the change observed in its mRNA accumulation. However, in streptozotocin-diabetic rat liver a distinct difference in the reduction of the individual alternative translation forms of C/EBPα was observed (section 2.3.1.3). There was a significant 50-60% decrease in the smaller 29 kDa form of C/EBPα, but only a moderate 25% decrease in the levels of the transcriptionally active, full length 42 kDa form of C/EBPα. Insulin treatment of the diabetic rat livers was seen to restore the levels of both C/EBPα translation products to control levels, if not slightly elevating the levels of the 42 kDa form over that of controls.

It should be noted that all results presented in section 4.1 of this thesis have been reported in Crosson, *et al.* (1997).

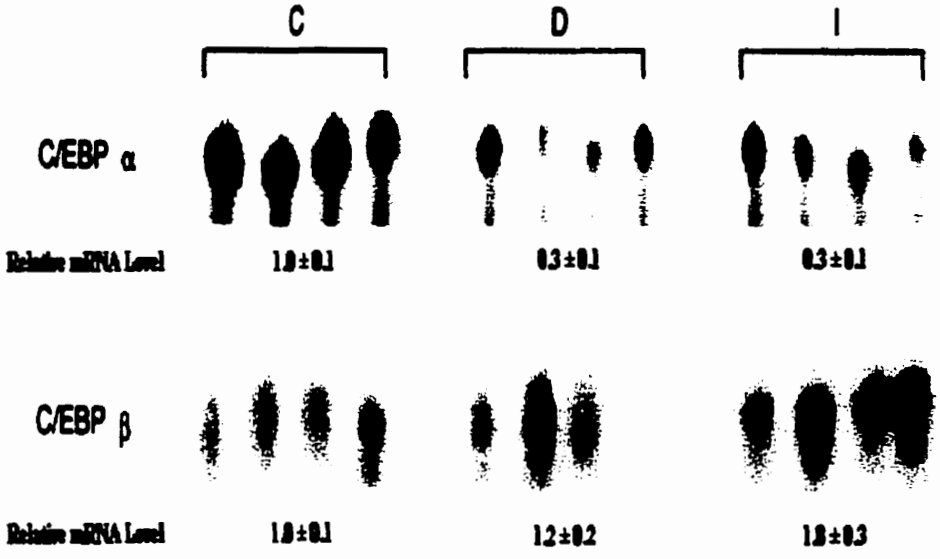

Figure 4.6 Effect of diabetes and insulin treatment on C/EBPα and C/EBPβ mRNA accumulation in rat liver. Rats were made diabetic and subsequently treated with insulin as outlined in section 3.4. Total RNA isolated from rat livers (section 3.7) was subjected to ribonuclease protection analysis (section 3.9). The abbreviations used are: C - saline controls (n=4); D - streptozotocin-diabetic rats (n=4); I - insulin-treated diabetic rats (n=4). The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown). Values shown are the means ± S.E..

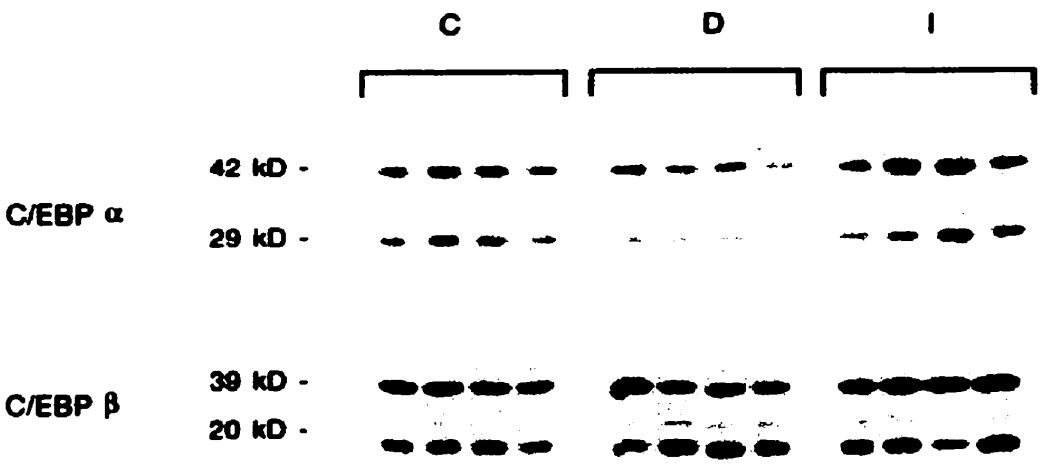

Figure 4.7 Effect of diabetes and insulin treatment on the levels of C/EBPα and C/EBPβ proteins in rat liver. Protein levels of C/EBPα, C/EBPβ were determined by Western analysis as outlined in section 3.13. The abbreviations used are: C - saline controls (n=4); D - streptozotocin-diabetic rats (n=4); I - insulin-treated diabetic rats (n=4). The equilibration of protein amount between samples was confirmed by assessing the levels of the general transcription factor TFIIEα (data not shown).

4.2 Characterization of Rat Hepatoma H4IIE Stable Cell Lines and the Effects of Inhibition of C/EBP Isoform Activity on PEPCK-C Gene Transcription

Having characterized the patterns of hormonal regulation of C/EBPα and C/EBPβ within the H4IIE cell line we next set out to characterize a role of C/EBP isoforms in the mediation of hormonally responsive gene expression. As discussed in section 2.2.3.1.3, the requirement of C/EBP for the basal and cAMP inducible expression of PEPCK-C has been well characterized. However, previous *in cyto* studies based on synthetic promoter systems and over expressed transcription factors have not been successful in identifying the specific C/EBP isoforms required to mediate particular transcriptional responses of the PEPCK-C gene. In fact recent work by Park, *et al.* (1999), has suggested that the two main hepatic C/EBP isoforms, α and β, can act interchangeably to mediate cAMP responsiveness when bound to the PEPCK-C promoter. While serving well to suggest the potential for a given factor(s) to mediate a certain transcriptional response, such synthetic-based experiments cannot reflect the molecular mechanisms which occur in an endogenous setting with absolute certainty. In order to address the nature of these endogenous mechanisms, a experimental system must be designed which allows the transcription factors of interest to be manipulated or otherwise altered so that their role in the transcriptional mechanisms of an endogenous promoter can be interpreted. Methodologies by which the activity of cellular proteins can be altered have been discussed in section 2.4. In order to determine the specific identity of the hepatic C/EBP isoform absolutely required to mediate the cAMP responsiveness of the endogenous PEPCK-C promoter, a number of

stable cell lines have been prepared which express specific molecules designed to inhibit generalized or specific C/EBP isoform activity (see sections 2.4.1 and 2.4.2).

H4IIE stable cell lines expressing either GBF-F, C/EBPα or C/EBPβ antisense RNA or C/EBPα sense RNA were prepared as outlined in section 3.16. A number of clones of each cell line were examined for the levels of expression of the specific gene of interest (data not shown). The strongest expressing clone was selected for further characterization and was thereafter propagated exclusively. As a matter of general note all results shown in the following sections are representative of at least three independent experiments and quantitative values presented are the averages of at least three experiments.

4.2.1 Characterization of the GBF-F D4 H4IIE Cell Line

In order to confirm the general requirement of C/EBP isoforms for PEPCK-C basal and cAMP inducible expression, a H4IIE cell line was prepared which expresses a dominant negative inhibitor of C/EBP isoform activity, GBF-F (see section 2.4.1). High levels of GBF-F expression within the intact cell should indiscriminately inhibit the transcriptional activity of all C/EBP isoforms by the formation of inactive heterodimers. The levels of GBF-F expression in the GBF-F D4 clone are shown in panel A of Fig. 4.8. Panel B of Fig. 4.8 shows a representative autoradiogram which demonstrates the effects of GBF-F expression on C/EBPα and C/EBPβ mRNA levels in the GBF-F D4 cell line in

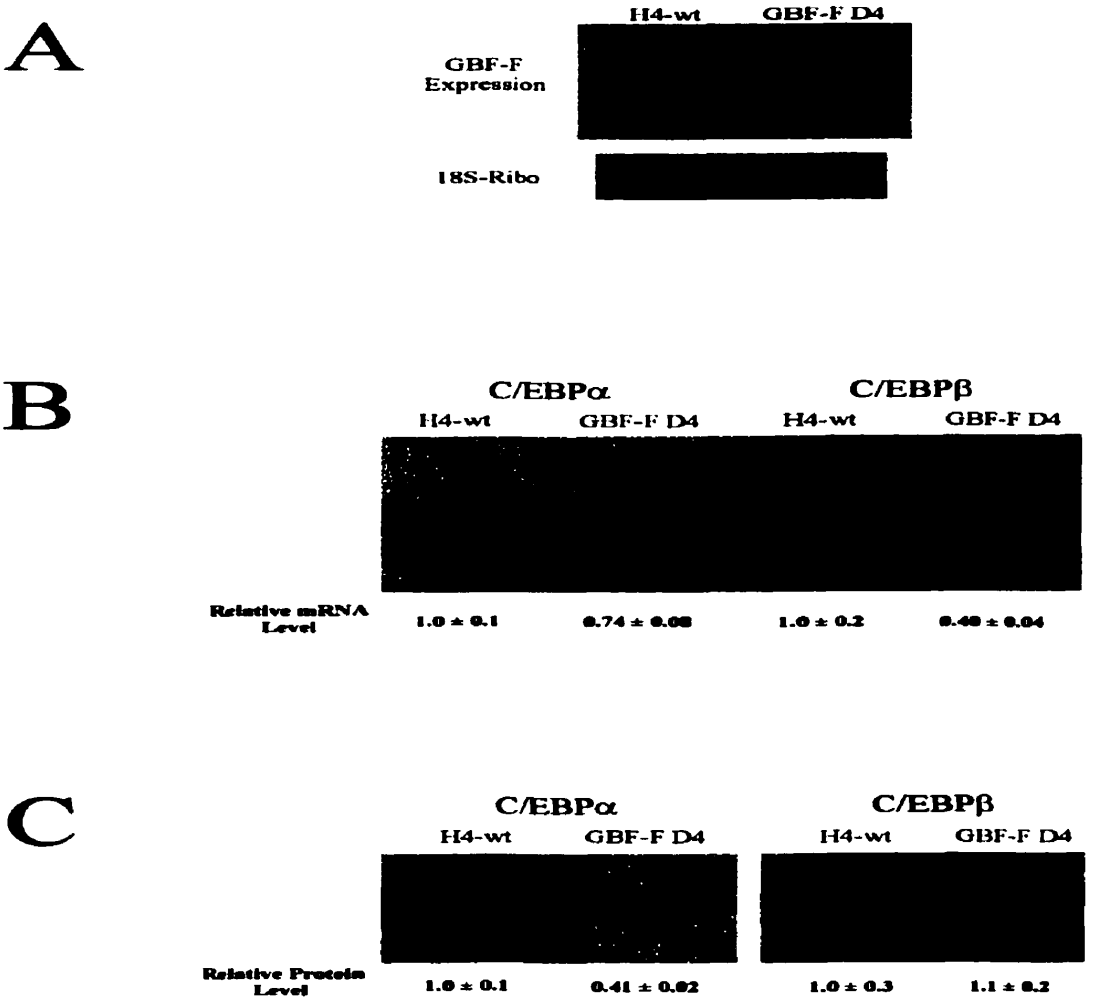

Figure 4.8 Comparison of wild type H4IIE cells (H4-wt) to a H4IIE stable transfected clone expressing GBF-F (GBF-F D4). Panel A is a representative Northern blot (section 3.11) showing GBF-F expression levels in the GBF-F D4 clone in comparison to H4IIE wild type cells. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12. Panel B shows a representative ribonuclease protection analysis (section 3.9) of C/EBPα and C/EBPβ mRNA levels in the GBF-F D4 clone in comparison to H4IIE wild type cells. Panel C shows a representative Western blot analysis (section 3.15) of C/EBPα and C/EBPβ protein levels in the GBF-F D4 clone in comparison to H4IIE wild type cells. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα. The results shown are representative of at least three independent experiments. Values reported are means ± S.E. of three independent experiments. C/EBPα and C/EBPβ mRNA and protein levels in wild type H4IIE cells were arbitrarily assigned a value of 1.

comparison to wild type H4IIE cells. The expression of GBF-F was observed to produce an approximate 30% reduction in C/EBPα mRNA levels and a 65% reduction in C/EBPβ mRNA levels. Despite the larger repressive effect of GBF-F expression upon C/EBPβ mRNA levels, this effect was not parallelled by its protein; no difference was detected in the levels of C/EBPβ protein between GBF-F D4 cells and wild type H4IIE cells (as shown in panel C of Fig. 4.8). However, the levels of C/EBPα protein were seen to be reduced by nearly 60% in GBF-F D4 cells in comparison to wild type H4IIE cells.

4.2.2 Characterization of the αA B1 H4IIE Cell Line

In order to produce a model system in which to test the requirement for the α-isoform of C/EBP in the hormonal responsiveness of the PEPCK-C promoter, a cell line expressing antisense RNA to C/EBPα was prepared. Panel A of Fig. 4.9 shows the levels of expression of the C/EBPα antisense RNA in the αA B1 H4IIE cell line in comparison to wild type H4IIE cells. As would be expected, the expression of an antisense RNA to C/EBPα within the αA B1 H4IIE cell line inhibited the overall expression of the transcription factor. As shown in panel B of Fig. 4.9, the levels of C/EBPα mRNA were reduced by 80% in the C/EBPα antisense expressing αA B1 cell line in comparison to wild type H4IIE cells. Most importantly from the aspect of functional inhibition of C/EBPα activity, the levels of C/EBPα protein were also decreased in the αA B1 cell line by nearly

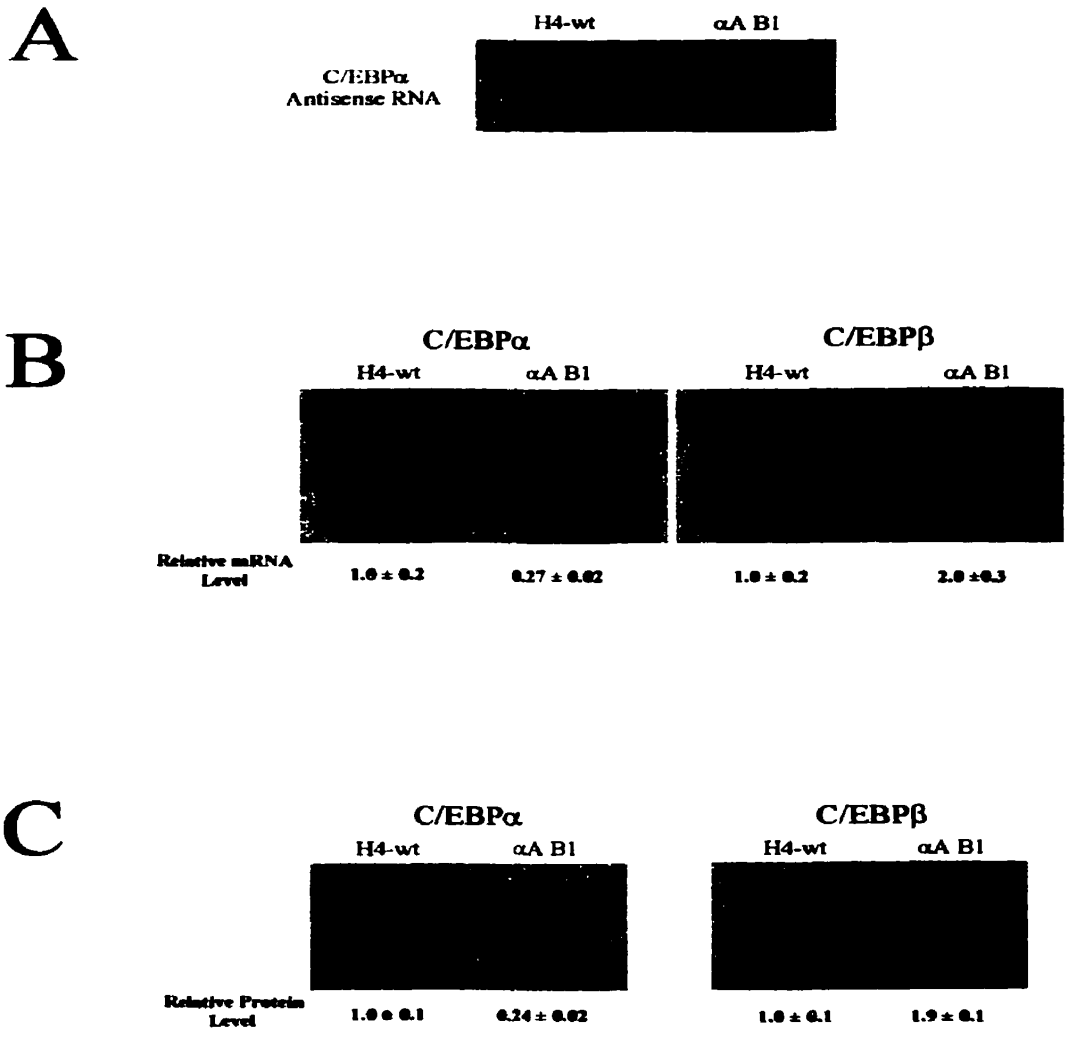

Figure 4.9 Comparison of wild type H4IIE cells (H4-wt) to a H4IIE stable transfected clone expressing C/EBPα antisense RNA (αA B1). Panel A is a representative ribonuclease protection analysis (section 3.9) showing C/EBPα antisense RNA expression in the αA B1 clone in comparison to H4IIE wild type cells. Panel B shows a representative ribonuclease protection analysis of C/EBPα and C/EBPβ mRNA levels in the αA B1 clone in comparison to H4IIE wild type cells. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown). Panel C shows a representative Western blot analysis (section 3.15) of C/EBPα and C/EBPβ protein levels in the αA B1 clone in comparison to H4IIE wild type cells. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα (data not shown). The results shown are representative of at least three independent experiments. Values reported are means ± S.E. of three independent experiments. C/EBPα and C/EBPβ mRNA and protein levels in wild type H4IIE cells were arbitrarily assigned a value of 1.

80% in comparison to wild type cells (refer to panel C of Fig. 4.9). An interesting dichotomy was observed between the effects of C/EBPα antisense RNA expression upon C/EBPα and C/EBPβ expression. The expression of C/EBPα antisense RNA caused an opposing effect on C/EBPβ expression in that it doubled both C/EBPβ mRNA and protein levels in comparison to levels in wild type H4IIE cells (panels B and C of Fig. 4.9). Thus it would appear that C/EBPα expression is significantly inhibited within αA B1 cells and that there is a corresponding increase in the expression of the β-isoform of C/EBP.

4.2.3 Characterization of the βA C4 H4IIE Cell Line

In order to assess the requirements for the β-isoform of C/EBP in hormonally regulated PEPCK gene expression, a stable H4IIE cell line was prepared which expresses antisense RNA to C/EBPβ. Panel A of Fig. 4.10 shows the level of expression of C/EBPβ antisense RNA in the βA C4 H4IIE cell line in comparison to H4IIE wild type cells. Parallelling the observations made in the C/EBPα antisense RNA expressing cell line αA B1, the expression of C/EBPβ antisense RNA in the βA C4 H4IIE cell line reduced the levels of C/EBPβ sense mRNA and protein relative to wild type H4IIE cells. The change in C/EBPβ mRNA in βA C4 cells as a consequence of C/EBPβ antisense RNA expression corresponded to a 45% reduction (Fig. 4.10, panel B).

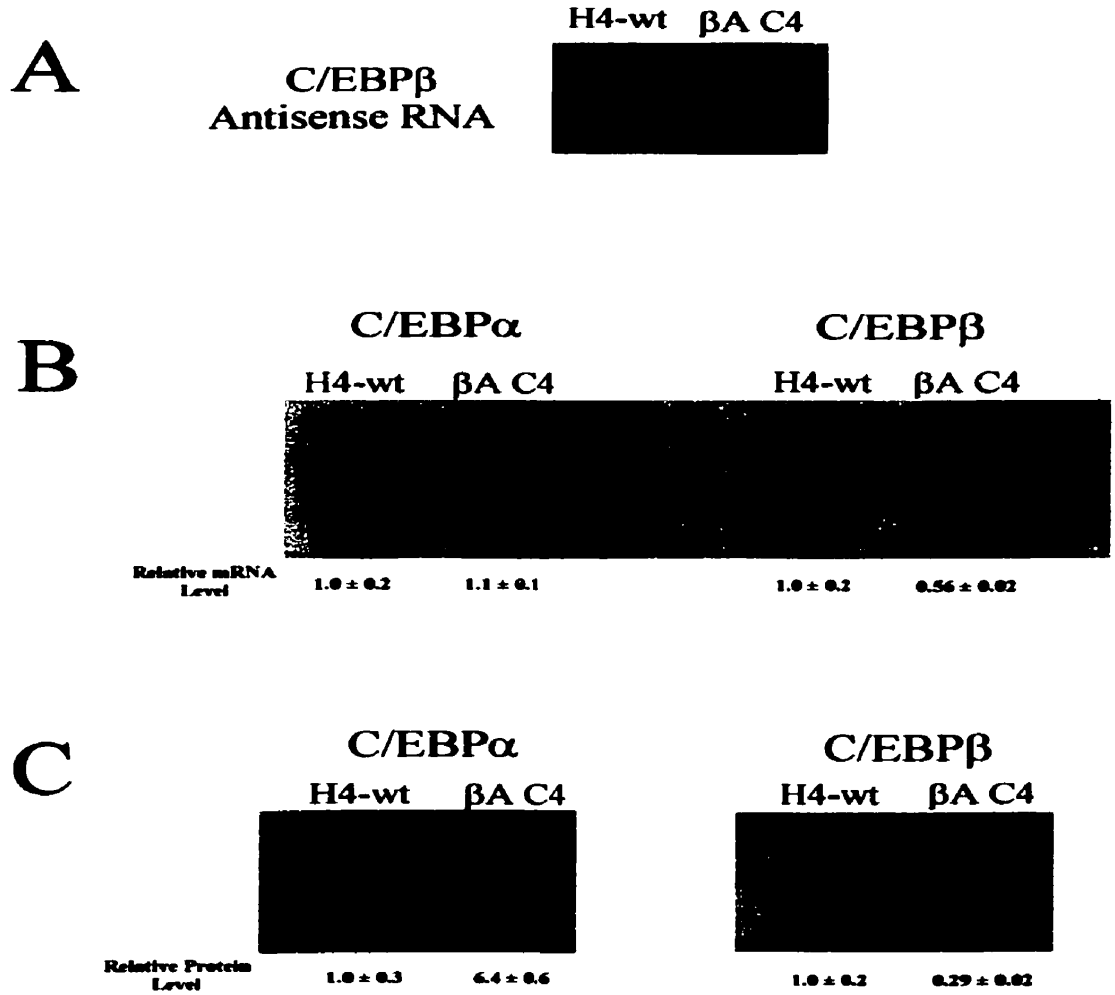

Figure 4.10 Comparison of wild type H4IIE cells (H4-wt) to a H4IIE stable transfected clone expressing C/EBPβ antisense RNA (βA-C4). Panel A is a representative ribonuclease protection analysis (section 3.9) showing C/EBPβ antisense RNA expression in the βA-C4 clone in comparison to H4IIE wild type cells. Panel B shows a representative ribonuclease protection analysis of C/EBPα and C/EBPβ mRNA levels in the βA-C4 clone in comparison to H4IIE wild type cells. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12. Panel C shows a representative Western blot analysis (section 3.15) of C/EBPα and C/EBPβ protein levels in the βA-C4 clone in comparison to H4IIE wild type cells. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα. The results shown are representative of at least three independent experiments. Values reported are means ± S.E. of three independent experiments. C/EBPα and C/EBPβ mRNA and protein levels in wild type H4IIE cells were arbitrarily assigned a value of 1.

A corresponding 70% reduction in C/EBPβ protein levels in comparison to wild type H4IIE cells was observed in the βA C4 cell line (Fig. 4.10, panel C). Thus the expression of C/EBPβ antisense RNA within the βA C4 cell line reduced the potential activity of C/EBPβ. Much like the observed dichotomy in C/EBPα and C/EBPβ expression patterns observed in the αA B1 cell line, a similar but opposite relationship occured in the βA C4 cell line as the result of C/EBPβ antisense RNA expression. The levels of C/EBPα protein in the βA C4 cell line were increased by 6-fold on average in comparison to wild type H4IIE cells (panel C of Fig. 4.10). Interestingly, unlike the parallel effect on C/EBPβ mRNA levels and protein observed in the αA B1 cell line, no change in C/EBPα mRNA levels were observed in the βA C4 cell line in comparison to wild type cells.

4.2.4 Characterization of the GαN175 A3 H4IIE Cell Line

In order to assess the effects of C/EBP isoform inhibition on gene expression utilizing a stable transfected cell model system, a suitable control must be produced. Such a control must account for possible changes in cellular physiology due to the process of stable integration of expression cassettes into the genome, as well as account for the possible effects upon cellular metabolism because of the selection process. A suitable control was produced by creating a H4IIE stable cell line expressing a sense C/EBPα RNA encoding for nucleotides +117 to +658. The levels of C/EBPα sense RNA expression in

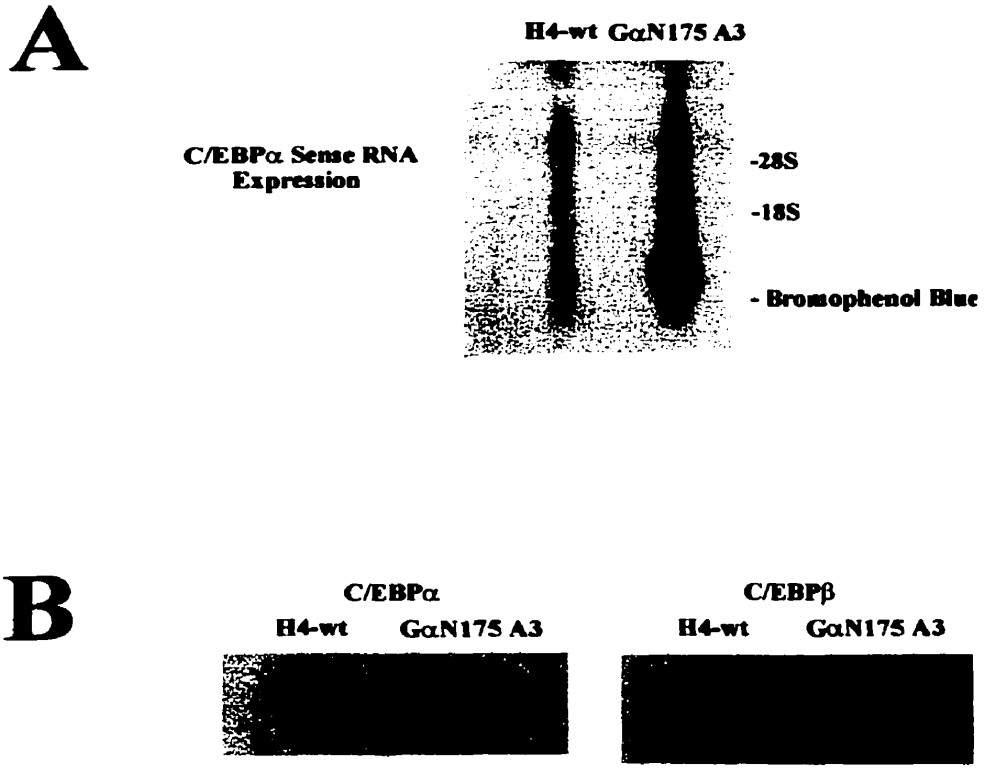

Figure 4.11 Comparison of wild type H4IIE cells (H4-wt) to a H4IIE stable transfected clone expressing a C/EBPα sense RNA construct (GαN175 A3). Panel A is a representative Northern blot (section 3.11) showing sense C/EBPα RNA construct expression levels in the GαN175 A3 clone in comparison to wild type H4IIE cells. The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using a 18S rRNA probe as described in section 3.12 (data not shown). Panel B shows a representative Western blot analysis (section 3.15) of C/EBPα and C/EBPβ protein levels in the GαN175 A3 clone in comparison to H4IIE wild type cells. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα (data not shown). The results shown are representative of at least three independent experiments.

the GαN175 A3 cell line in comparison to wild type H4IIE cells is shown in panel A of Fig. 4.11. As shown by representative autoradiograms in panel B of Fig. 4.11, no significant difference in C/EBPα or C/EBPβ protein levels was detected in the GαN175 A3 cell line in comparison to wild type H4IIE cells as a consequence of the expression of the GαN175 C/EBPα sense RNA.

4.2.5 Effect of C/EBP Isoform Inhibition on PEPCK-C cAMP Responsiveness

The specific goal in producing the aforementioned stable H4IIE cell lines was to determine the identity of the C/EBP isoform required to mediate the cAMP responsiveness of the endogenous PEPCK-C promoter. Having characterized the expression of GBF-F, C/EBPα antisense RNA or C/EBPβ antisense RNA in these cell lines (sections 4.2.1 thru 4.2.3), and having determined that the antisense expressing lines exhibit significant reduction in the levels of the targeted C/EBP isoform protein (Figs. 4.9 and 4.10), we next set out to assess the effects of this inhibition on PEPCK-C cAMP responsiveness. Figure 4.12a displays representative autoradiograms and Figure 4.12b shows a graphical representation of three independent ribonuclease protection analysis designed to test the C/EBP requirements for PEPCK-C cAMP responsiveness.

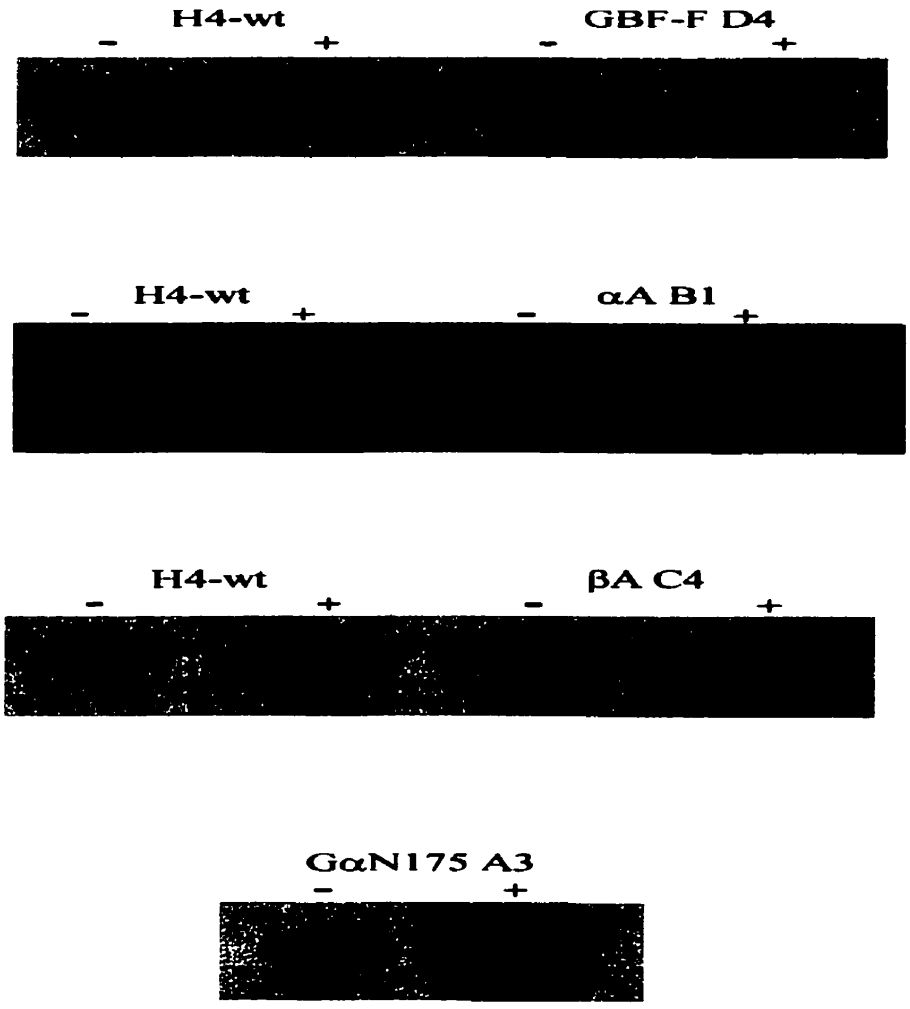

Figure 4.12a The effects of GBF-F, C/EBPα antisense, C/EBPβ antisense or C/EBPα sense RNA on endogenous PEPCK-C cAMP responsiveness in stable H4IIE cell lines in comparison to wild type H4IIE cells. Changes in PEPCK-C mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. Wild type H4IIE or stable H4IIE cell lines were treated with 200 μM 8-CPT-cAMP for 4 hours (+); controls are designated as (-). The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown). The above panels are representative of at least three independent experiments. Quantitation of the changes in PEPCK mRNA levels in each cell line relative to untreated H4-wt cells are reported in Figure 4.12b.

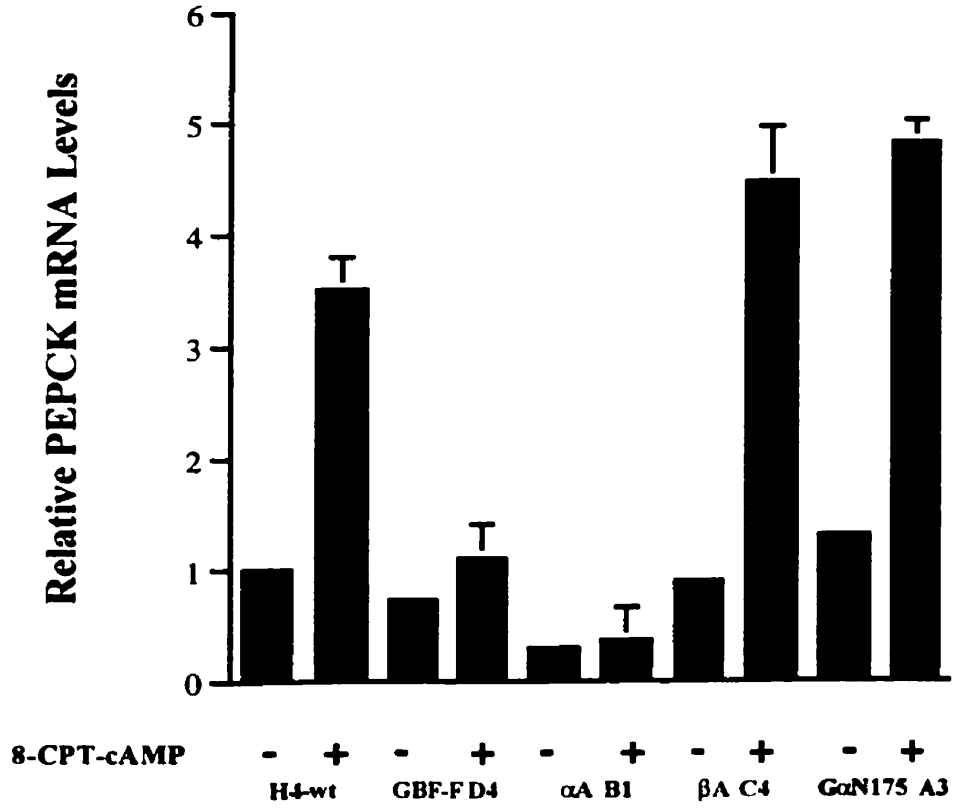

Figure 4.12b Graphical representation of the effects of GBF-F (GBF-F D4), C/EBPα antisense RNA (αA B1), C/EBPβ antisense RNA (βA C4) or C/EBPα sense RNA (GαN175 A3) on endogenous PEPCK-C cAMP responsiveness in stable H4IIE cell lines in comparison to wild type H4IIE cells (H4-wt). Changes in PEPCK mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. Representative autoradiogram have been shown in Figure 4.12a. Wild type H4IIE or stable H4IIE cell lines were treated with 200 μM 8CPT-cAMP for 4 hours (+); controls are designated as (-). The above graph displays the means ● S.E. of three independent experiments.

In wild type H4IIE cells, a 4 hour 200 μM 8-CPT-cAMP treatment produced an approximate 3-fold increase in PEPCK-C mRNA accumulation. The responsiveness of PEPCK-C to 8-CPT-cAMP was abolished in the GBF-F expressing cell line GBF-F D4, confirming the general requirement of C/EBP isoforms in cAMP responsiveness. Furthermore, the general inhibition of C/EBP isoform activity within the GBF-F D4 cell line also affected PEPCK-C basal expression, with a 35% reduction in PEPCK-C mRNA levels in GBF-F D4 cells in comparison to wild type H4IIE cells.

The inhibition of C/EBPα activity by antisense within the αA B1 cell line also abolished PEPCK-C cAMP responsiveness (Fig. 4 .12). The levels of PEPCK-C basal expression were also reduced in the αA B1 cell line, where a 70% reduction was observed in comparison to wild type cells.

An inhibition of PEPCK-C cAMP responsiveness was not observed in C/EBPβ antisense RNA expressing βA C4 cells (Fig. 4.12). In fact, the fold induction of PEPCK-C mRNA levels within the βA C4 cell line was routinely observed to be greater than that seen in wild type H4IIE cells, producing a 5-fold increase compared with the 3-fold induction observed in wild type H4IIE cells. No significant difference in PEPCK-C basal expression levels were observed in βA C4 cells relative to H4IIE wild type.

As expected, no inhibition of PEPCK-C cAMP responsiveness was observed in the C/EBPα sense RNA expressing cell line GαN175 A3 (Fig. 4.12). Thus, the processes of stable integration and selection appeared not to have interfered with the molecular mechanisms of cAMP induction of endogenous PEPCK-C within the stable transformed cell lines.

From this data, it is concluded that C/EBPα is the specific C/EBP isoform which along with the other characterized CRU binding *trans*-acting factors, together mediate the cAMP induction of the endogenous PEPCK-C gene with the H4IIE rat hepatoma cell.

4.2.6 Effect of C/EBPα Antisense RNA and GBF-F on Cellular cAMP Signalling

In order to properly interpret the results presented in section 4.2.5 regarding the effects of the inhibition of C/EBP isoform activity upon PEPCK-C cAMP responsiveness, the possibility that C/EBPα inhibition exerted its effects in an indirect fashion, perhaps by affecting the levels of a protein required for general cAMP responsiveness such as CREB needed to be addressed. Thus, it was necessary to determine whether cAMP responsiveness in general was reduced, or whether it was only cAMP responsiveness requiring the presence of C/EBPα affected. The levels of CREB protein were not significantly altered in the GBF-F D4 or αA B1 cell lines relative to wild type cells (data not shown). The effects of GBF-F and C/EBPα antisense RNA expression upon the cAMP responsiveness of a endogenous H4IIE gene other than PEPCK-C were also assessed. As the results displayed in Figs. 4.2 and 4.4 have shown, the gene for C/EBPβ is acutely affected by 8-CPT-cAMP treatment at the levels of both mRNA and protein. The cAMP inducibility of the C/EBPβ gene is believed to require only the mediation of CREB (Niehof, *et al.* 1997). In order to determine whether C/EBPβ protein could still be induced by 8-CPT-cAMP in the αA B1 and GBF-F D4 cell lines these cells were treated with 1 mM

8-CPT-cAMP for 8 hours, parallelling the experiment performed in wild type H4IIE cells shown in Fig. 4.4. The effect of 8-CPT-cAMP treatment of these cell lines upon C/EBPβ protein was assessed by Western analysis as shown in Fig. 4.13. The levels of C/EBPβ protein were induced by 3-fold on average in both αA B1 and GBF-F D4 cells, which is comparable to the induction observed in wild type H4IIE cells shown in Fig. 4.4. Thus it would appear that overall cellular cAMP signalling in the C/EBPα antisense RNA and GBF-F expressing cell lines is operating in a manner comparable to that of wild type H4IIE cells. This result would suggest that the abolishment of PEPCK-C cAMP responsiveness in the C/EBPα antisense RNA and GBF-F expressing cell lines is a unique effect due to the down-regulation of C/EBP isoform activity and is not due to some general suppression of the cAMP signalling pathway.

4.2.7 Effect of C/EBP Isoform Inhibition on PEPCK-C Glucocorticoid Responsiveness

As C/EBP isoforms have been implicated in having accessory factor function in the responsiveness of the PEPCK-C promoter to glucocorticoids (see section 2.2.3.1.1), the next avenue of investigation involved the characterization of the C/EBP isoform requirements in the glucocorticoid responsiveness of endogenous PEPCK-C. As shown in Fig. 4.14, cells expressing GBF-F, C/EBPα or C/EBPβ antisense RNA were treated with 1 μM dexamethasone for 8 hours and the levels of induction of PEPCK-C mRNA

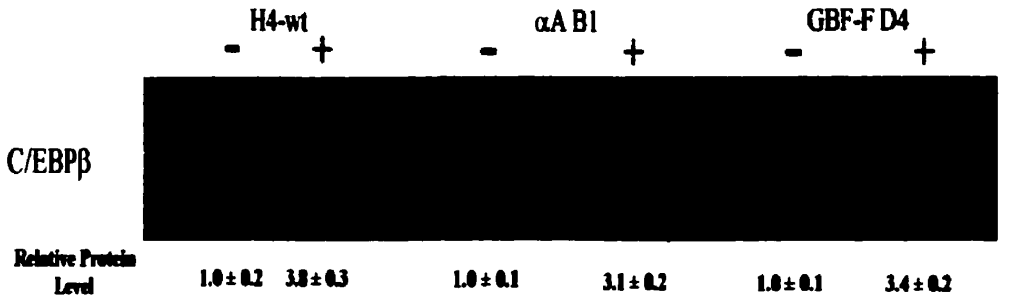

Figure 4.13 The effects of C/EBPα antisense RNA or GBF-F expression upon C/EBPβ cAMP responsiveness in stable H4IIE cell lines. H4IIE stable cell lines expressing either C/EBPα antisense RNA (αA B1) or GBF-F (GBF-F D4) were treated with 1 mM 8-CPT-cAMP for 8 hours (+); controls are designated as (-). C/EBPβ protein levels were assessed by Western analysis as outlined in section 3.15. The equilibration of protein amount between samples was confirmed by assessing the protein levels of the general transcription factor TFIIEα (data not shown). The panel shown is representative of at least three independent experiments. Values reported are means ± S.E. of three independent experiments. The levels of C/EBPβ protein in untreated cells were arbitrarily assigned a value of 1.

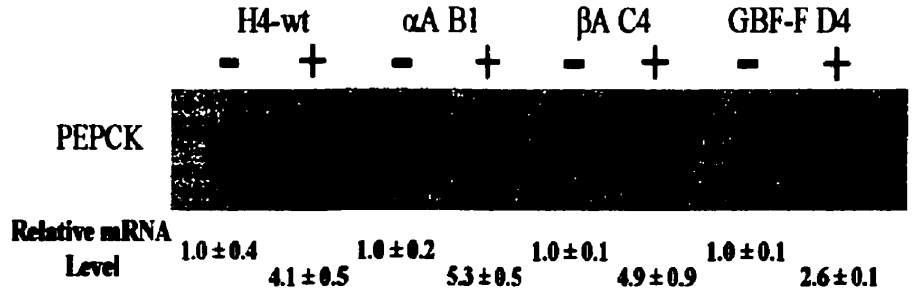

Figure 4.14 The effects of C/EBPα antisense RNA, C/EBPβ antisense RNA or GBF-F expression on PEPCK-C glucocorticoid responsiveness in H4IIE stable cells lines in comparison to H4IIE wild type cells. Changes in PEPCK-C mRNA accumulation were assessed by ribonuclease protection analysis as outlined in section 3.9. Wild type H4IIE or stable H4IIE cell lines were treated with 1 μM dexamethasone for 8 hours (+); controls are designated as (-). The equilibration of RNA amounts between samples was verified by RNA slot-blot analysis using an 18S rRNA probe as described in section 3.12 (data not shown). The above panel is representative of at least three independent experiments. Values reported are means ± S.E. of four independent experiments. The levels of PEPCK mRNA in untreated cells were arbitrarily assigned a value of 1.

were compared to that occurring in similarly treated wild type H4IIE cells. The levels of PEPCK-C mRNA induction in response to dexamethasone were comparable in C/EBPα and C/EBPβ antisense RNA expressing cells to that in wild type H4IIE cells, with a 5-fold induction observed on average in these cell types. However, in GBF-F expressing cells the level of dexamethasone induction of PEPCK-C mRNA was somewhat inhibited in comparison to wild type cells, with the level of induction averaging out to approximately 2.5-fold.

5. DISCUSSION

The regulation of gene expression at the level of transcription is a complex and multi-tiered phenomenon. Not only are genes regulated by various environmental signals transduced through cell-signalling pathways, which are ultimately mediated by nuclear transcription factors, but it is now known that the expression of transcription factors themselves is intricately regulated.

One of the objectives of this research project was to contribute to the general understanding of how the expression of transcription factors is regulated. Specifically, the goal was to characterize the unique patterns of regulation of the two main hepatic C/EBP isoforms by glucose homeostasis-affecting hormones, as well as to establish which C/EBP isoform mediates the effects of specific hormonal signals on the gene for PEPCK-C, which codes for an enzyme central to glucose homeostasis.

As the results of this thesis work have shown, the expression of C/EBP isoforms is regulated by glucose homeostasis-affecting hormones in the liver, and the hormonal regulation of C/EBPα in particular appears to be distinct from that which occurs in other tissues examined to date. Furthermore, this work demonstrates the *in cyto* requirement for a specific C/EBP isoform in the mediation of the cAMP responsiveness of the endogenous PEPCK-C gene, and has also generated a model cell system which can be utilized for the

analysis of C/EBP isoform requirements in the mediation of various environmental signals upon the endogenous PEPCK-C gene, or potentially any other hepatic gene of interest.

5.1 Hormonal Regulation Studies

5.1.1 The Hormonal Regulation of C/EBPα in Liver

Experiments designed to characterize the patterns of hormonal regulation of C/EBP isoforms in H4IIE cells revealed that glucocorticoids are a dominant regulator of both C/EBPα and C/EBPβ expression (Figures 4.2 and 4.4). The effects of dexamethasone on C/EBPα expression in H4IIE cells appear to be cell specific. Whereas dexamethasone induces both C/EBPα mRNA accumulation and protein levels in H4IIE cells (Figures 4.2. and 4.4), it has been shown to rapidly, albeit transiently, reduce the levels of C/EBPα in 3T3-L1 adipocytes and in white adipose tissue (MacDougald, *et al.* 1994). Interestingly, this reciprocal action of dexamethasone on gene expression between liver and adipose tissue is also witnessed in the regulation of PEPCK-C gene expression (Meisner, *et al.* 1985 and Nechushtan, *et al.* 1987). In studies utilizing the IEC-6 rat intestinal crypt cell line, dexamethasone had no observable effect upon C/EBPα expression patterns (Boudreau, *et al.* 1996). Thus it would appear that the regulation of C/EBPα by glucocorticoids is uniquely tailored to the tissue in which it is expressed, perhaps based upon the metabolic roles of the constituent genes which it *trans*-activates.

In addition to the effects of glucocorticoids on the expression of hepatic genes critical for metabolic processes, these steroid compounds are also known to inhibit cell

growth and division in regenerating liver and in some liver-derived cell lines (Cook, *et al.* 1988). In correlation with our own findings, Ramos, *et al.* (1996), demonstrated that the dexamethasone-induced expression of C/EBPα is required for the glucocorticoid-mediated cell cycle arrest of minimal deviation rat hepatoma cells.

8-CPT-cyclic-AMP was observed to have relatively minor effects on C/EBPα expression both *in cyto* and *in vivo* in comparison to its effects on C/EBPβ (Figures 4.2 and 4.5). The effects of glucagon, *via* cAMP, on C/EBPα expression have not been previously reported. While the induction of C/EBPα mRNA accumulation by 8-CPT-cAMP was negligible, and non-existent within adult rat liver, a small but significant induction of C/EBPα protein levels was routinely observed in H4IIE cells. Such a lack of correlation between C/EBP isoform mRNA and protein levels is not uncommon and has been reported by Descombes, *et al.* (1990), who observed large differences in the ratios of C/EBPβ mRNA/protein levels in liver and in lung. In that study, liver tissue was seen to have lower levels of C/EBPβ mRNA than lung, yet liver accumulated significant amounts of C/EBPβ protein relative to lung tissue. A similar phenomenon has been observed with another structurally related transcription factor, the D-site binding protein (DBP), whose mRNA can be detected in many different tissues and yet whose protein can only be detected in significant amounts in liver (Mueller, *et al.* 1990). Thus post-transcriptional regulation of transcription factor expression is not uncommon, and can often account for differences observed between the levels of mRNA and protein.

The effects of insulin on C/EBPα expression in H4IIE cells was bi-phasic, initially inhibiting and then inducing its levels of mRNA in a temporal fashion (Figure 4.2). This

observation was extended to include parallel effects on C/EBPα protein (Figure 4.4). The effects of insulin on C/EBPα expression in combination with other hormones was varied and seemed dependent upon the hormonal combination. The effects of insulin on C/EBPα expression in intestinal tissue can only be inferred indirectly from experiments involving serum treatment of rat intestinal crypt IEC-6 cells by Boudreau, *et al.* (1996). This study demonstrated a small but significant increase in C/EBPα mRNA levels by some post-transcriptional mechanism. The reported effects of insulin upon C/EBPα expression in fully-differentiated 3T3-L1 adipocytes appear similar to its effects in the liver cell; however it appears to act far more acutely (MacDougald, *et al.* 1995). Differentiated 3T3-L1 cells treated with large doses of insulin exhibited a dramatic decrease in C/EBPα protein levels within 4 hours, whereas the results presented in Figure 4.4 showed that the insulin-induced decrease in C/EBPα protein is not observable in H4IIE cells until a 16 hour time point, albeit at a lower concentration of insulin. Thus it would appear that much like the effects of glucocorticoids upon C/EBPα expression, the effects of insulin upon this transcription factor vary between different tissues, which again could be based on the metabolic profile of the tissue and the tissue-specific properties of the genes which C/EBPα is called upon to *trans*-activate.

5.1.2 The Hormonal Regulation of C/EBPβ in Liver

In general, the effects of a given hormonal treatment on C/EBPβ mRNA or protein levels were much more significant than those on C/EBPα expression in regards to the fold-changes observed. As discussed in section 5.1.1, glucocorticoids were observed to have a dominant inductive effect on the expression of C/EBPβ (Figures 4.2 and 4.4), much like what was observed with C/EBPα, although this inductive effect was more profound. Our results correlate with the findings of Matsuno, *et al.* (1996), who demonstrated a similar effect of dexamethasone upon C/EBPβ mRNA accumulation in primary-cultured rat hepatocytes. However, it appears that the effects of glucocorticoids on C/EBPβ expression in the primary hepatocyte is far more acute and transient than its effects in the H4IIE rat hepatoma, as Matsuno, *et al.* (1996), observed dexamethasone-induced induction of C/EBPβ mRNA within 30 min, which peaked at 2 hours and then gradually decreased. Another report concerning the effects of glucocorticoids upon C/EBPβ expression was conducted in an intestinal cell line by Boudreau, *et al.* (1996). This study revealed an inductive effect on C/EBPβ mRNA and protein levels in these cells in response to dexamethasone treatment which could be accounted for by changes in the rate of transcriptional initiation of the C/EBPβ gene.

8-CPT-cyclic-AMP had a large inductive effect on C/EBPβ mRNA levels both *in cyto* and *in vivo* (Figures 4.2 and 4.5), and a significant acute inductive effect on C/EBPβ proteins levels in H4IIE cells (Figure 4.4). A similar effect of cAMP on hepatic C/EBPβ mRNA levels *in vivo* was reported by Park, *et al.* (1993), utilizing similar experimental

conditions, and by Matsuno, *et al.* (1996), in rat primary hepatocytes. Cyclic-AMP is also known to strongly induce C/EBPβ mRNA accumulation in primary cultures of Sertoli cells (Gronning, *et al.* 1999). The effects of cAMP on C/EBPβ expression in other tissues has not been reported.

Insulin was seen to have an inductive effect on both C/EBPβ mRNA accumulation and protein levels in H4IIE cells (Figures 4.2 and 4.4). This observation correlates with the observed insulin-mediated induction of C/EBPβ mRNA and protein levels in rat intestinal IEC-6 cells (Boudreau, *et al.* 1996), and in fully differentiated 3T3-L1 cells (MacDougald, *et al.* 1995). However, a report by Bosch, *et al.* (1995), contrasts with our findings, demonstrating an insulin-mediated decrease in C/EBPβ protein levels in adult mouse liver. Although our own results have demonstrated a parallel increase in both C/EBPβ mRNA and protein levels in response to prolonged insulin treatment *in cyto*, the results of Bosch, *et al.* (1995), may more clearly reflect the effects of insulin on the C/EBPβ gene product *in vivo*.

Thus, it appears that the effects of certain glucose homeostasis-affecting hormones upon C/EBPβ expression are conserved between different metabolically relevant tissues, unlike the uniquely tissue-specific regulation of C/EBPα by the same hormones. These observations offer support to the hypothesis presented by McKnight, *et al.* (1989), who have suggested that the α-isoform of C/EBP is a central regulator of energy homeostasis. If in fact C/EBPα is such a "central regulator", its expression patterns would have to be uniquely tailored to suit the metabolic role of a given tissue in regards to the

overall hormonal environment within the organism at any given time. Regulation of C/EBPα expression would have to occur such that it would be capable of the *trans*-activation of metabolically relevant genes within appropriate tissue-specific and temporal guidelines.

5.1.3 The Effects of Streptozotocin-Diabetes on C/EBPα Expression

Experiments designed to ascertain the effects of an experimental type I diabetic-like state on hepatic C/EBP isoform expression revealed a complex alteration in the expression of C/EBPα in diabetic rat liver (Figures 4.6 and 4.7). While streptozotocin diabetes was observed to decrease hepatic C/EBPα mRNA levels (Figure 4.6), an effect which was partially reversible by insulin treatment, the effects upon the two major translation forms of C/EBPα (section 2.3.1.3) were diverse (Figure 4.7). The levels of the 29 kDa form of C/EBPα were decreased relative to the full-length 42 kDa protein form in diabetic livers, suggesting a shift towards the preferential usage of the first AUG of the C/EBPα mRNA to initiate translation by an unknown molecular mechanism. A decrease in C/EBPα protein levels has also been observed in the kidneys of streptozotocin-treated rats, although no information regarding changes in specific C/EBPα translation forms was presented in that particular study (Zador, *et al.* 1998). A study similar to our own by Valera, *et al.* (1993), contradicted our findings, reporting a small increase in C/EBPα mRNA levels in rat streptozotocin-diabetic liver. A possibility for this inconsistency could be the differences

in the streptozotocin dose utilized to induce the diabetic state, or possibly differences in the severity and/or duration of the diabetes. It should be noted that our own findings demonstrated correlating changes in both C/EBPα mRNA and protein levels, whereas the work of Valera, *et al.* (1993), only examined diabetes-induced changes in C/EBPα mRNA levels.

Given the differences in *trans*-activational ability between the two C/EBPα translation forms (Friedman and McKnight (1990) and Pei and Shih (1991)), an increase in the ratio of one form to the other could have a significant effect on the overall *trans*-activational ability of C/EBPα in the streptozotocin-diabetic rat liver. The 29 kDa translation form of C/EBPα lacks the N-terminal 117 residues of the full-length form, which corresponds to the *trans*-activation domain, and thus is transcriptionally less active. The C/EBPα 29 kDa form does, however, possess the DNA-binding and dimerization domains, which allows it to form less active heterodimers with full-length 42 kDa C/EBPα. Given the increase in the ratio of active 42 kDa C/EBPα to inactive 29 kDa C/EBPα in streptozotocin-diabetic rat liver, an overall increase in the *trans*-activational activity of C/EBPα in diabetic liver could potentially occur.

It should be noted that distinct differences in C/EBP isoform translation form expression have also been observed under acute phase response conditions (An, *et al.* 1996), and in 3T3-L1 cells after treatment with thiazolidinediones (Hemati, *et al.* 1998). Although recent work by Lincoln, *et al.* (1998), suggested that the generation of truncated C/EBPα and C/EBPβ translation forms is due to proteolytic degradation and not due to a

translational start site multiplicity model, our own results and those of An, *et al.* (1996), and Hemati, *et al.* (1998), suggest a physiological role for alternate C/EBP translation forms. In addition, our own experience has shown that although truncated forms of C/EBPα and C/EBPβ are expressed at low levels within H4IIE cells, these alternate forms can still by detected by Western analysis when using similar lysis buffer conditions as those utilized by Lincoln, *et al.* (1998), which should otherwise prevent the formation of proteolytic products (data not shown). These findings suggest that the relative levels of the different C/EBP translation forms can be differentially regulated by changes in cellular physiology, and that these changes may play a role in defining the *trans*-activational properties of C/EBP isoforms in these altered physiological states.

Due to the complex nature of most gene promoters, it is difficult to make generalized statements regarding the consequences of altered C/EBPα expression during diabetes on overall hepatic gene expression and any possible correlation with the metabolic and physiological perturbations which occur in this disease state. Although it is difficult to assess how the changes in expression of this single transcription factor during diabetes might affect the overall physiological state of the organism, it is certain that C/EBPα does play a pivotal role in metabolism. Knockout of the C/EBPα gene has demonstrated the absolute requirement of this transcription factor for proper glycogen metabolism and developmental expression of pace-setting gluconeogenic enzymes including PEPCK-C (Wang, *et al.* 1995). Disruption of the *c/ebpα* gene in adult mice by the loxP / Cre recombinase methodology has also demonstrated the requirement of this factor in bilirubin detoxification and gluconeogenesis (Lee, *et al.* 1997). Work by Roesler, *et al.* (1996 and

1998b), as well as results presented in section 4.2 of this thesis, have demonstrated the requirement of C/EBPα in the maintenance of basal expression and mediation of the cAMP responsiveness of PEPCK-C gene transcription. Thus it appears that C/EBPα is an important regulator of hepatic carbohydrate metabolism, and changes in its expression which occur in streptozotocin-diabetes could have significant effects upon these metabolic processes which may ultimately contribute to the overall diabetic phenotype.

5.2 Discussion of PEPCK-C Gene Expression - C/EBP Isoform Requirement Studies

Experiments designed to determine the C/EBP isoform absolutely required to mediate the cAMP responsiveness of endogenous PEPCK-C in hepatoma cells have identified C/EBPα as this essential factor (Figure 4.12). Furthermore, the general requirement for C/EBP isoforms in the mediation of the PEPCK-C glucocorticoid response, potentially as accessory factors, has been confirmed. It would also appear that the exact identity of the C/EBP isoform required to participate in the PEPCK-C glucocorticoid response is functionally redundant based on the representative data presented in Figure 4.14. In order to conduct the above experimental work a number of rat hepatoma H4IIE stable cell lines were produced which serve as model systems to study the effects of general or specific C/EBP isoform inhibition upon endogenous gene expression and regulation.

5.2.1 The Effects on C/EBP Isoform Expression in Stable Rat Hepatoma H4IIE Cell Lines

5.2.1.1 The Effects of Stable GBF-F Expression on C/EBP Isoform Expression

The stable expression of the C/EBP isoform dominant negative molecule GBF-F appears to have significant effects on C/EBP isoform gene expression, most especially on that of C/EBPα (Figure 4.8). While affecting the mRNA levels of both hepatic C/EBP isoforms, the stable expression of GBF-F appears to have effects on the protein level of the α-isoform only. Unlike C/EBPα protein, C/EBPβ protein appears unaffected by stable GBF-F expression. As has been mentioned previously, it is not uncommon for differential regulation of C/EBP isoform mRNA and protein levels to be observed (section 5.1.1). No published data has been reported to date which supports the concept of transcriptional regulation of the C/EBPβ gene by C/EBP isoforms, although this possibility is not unlikely considering the known regulation of the C/EBPα gene promoter by C/EBP isoforms.

The observation that GBF-F stable expression inhibited overall C/EBPα expression is not particularly surprising given the recognized auto-regulation of the C/EBPα gene in mouse and human (section 2.3.2). The murine C/EBPα promoter is known to bind the C/EBPα protein directly (Christy, *et al.* 1991 and Legraverend, *et al.* 1993), and in transient transfection studies expression of C/EBPα has been shown to *trans*-activate co-expressed C/EBPα promoter-luciferase reporter constructs. The studies of Legraverend, *et al.* (1993), have also shown *trans*-activation of similar promoter constructs when co-

transfected with C/EBPβ expression vector but to a far lesser extent than experiments conducted with C/EBPα vectors. The work of Timchenko, *et al.* (1995), has also suggested a role of C/EBPα in auto-regulation of its own gene in humans. However, this study concluded that there was no direct binding of the C/EBPα protein to its promoter, rather that its effects were in some manner exerted through another binding protein termed the upstream stimulatory factor (USF). In whatever event, the auto-regulatory effects of C/EBPα on its own promoter have been established in two different species; unfortunately no information regarding the regulatory properties of the C/EBPα gene in rat has been reported. It would not, however, seem unlikely that similar mechanisms could occur in the rat and the results presented in section 4.2.1 of this thesis support this hypothesis. Given the down regulation of C/EBPβ mRNA accumulation in the GBF-F D4 cell line, it is also possible to hypothesize that the promoter of the rat C/EBPβ gene may also be responsive to the *trans*-activational properties of C/EBP isoforms.

An important consideration in the interpretation of the results of section 4.2.1 is a possible flaw in the design of the dominant negative GBF-F molecule. It has been suggested that although it is certainly capable of forming inactive heterodimers with C/EBP isoforms, the GBF-F molecule may also form similar inactive heterodimers with fos and jun (personal communication between W.J. Roesler and C.R. Vinson). Although unlikely to cause potential misinterpretation of data which suggest effects on the expression of C/EBPα by GBF-F expression, as no known fos/jun binding sites are thought to occur in the C/EBPα promoter, non-specific inhibition of fos/jun as well as C/EBP isoforms could

effect interpretation of data regarding its affects on the expression and regulation of other genes, including PEPCK-C. This concept will be considered further in section 5.2.2.

5.2.1.2 The Effects of Stable C/EBPα Antisense RNA on C/EBP Isoform Expression

The stable expression of specific antisense RNA to the α–isoform of C/EBP in the αA B1 H4IIE cell line appears to have significant effects upon the expression of both major hepatic C/EBP isoforms (Figure 4.9). Expression of C/EBPα antisense RNA had potent effects on both C/EBPα mRNA and protein levels. The approximately 80% reduction in C/EBPα mRNA and protein levels in the αA B1 cell line in comparison to wild type H4IIE cells is comparable to the inhibition observed by Lin and Lane, (1992), when they expressed a similar C/EBPα antisense RNA in 3T3-L1 adipocytes. The segment of the C/EBPα sequence utilized to produce antisense RNA by Lin and Lane, (1992), was chosen so as to insure as much specificity for the α-isoform of C/EBP as possible. The approximately 400 bp region of C/EBPα sequence utilized by these researchers corresponded to the *trans*-activation domain and was positioned far 5' to the conserved basic region and leucine zipper of C/EBP isoforms. The region of C/EBPα sequence utilized in this thesis work was approximately 250 bp larger, but corresponded to an encompassing region of sequence which was still unique to the α-isoform of C/EBP. Correlating with our observations, the work of Lin and Lane, (1992), also demonstrated an inhibition in the levels of C/EBPα mRNA as a result of antisense expression. This

inhibition was explained by these workers as being a consequence of their measured inhibition of the transcription rate of the C/EBPα gene. These workers postulated that this inhibition of C/EBPα transcription rate was perhaps brought about by a lack of auto-regulation by its own protein as a consequence of the antisense expression which could bring about decreased protein levels *via* an increased turnover of C/EBPα mRNA or inhibition of C/EBPα mRNA translation. This work, which appeared prior to the description of the auto-regulation of the C/EBPα gene, was the first instance of such a phenomenon being proposed. Interestingly, although never displaying any data, the work of Lin and Lane, (1992), also suggested that the expression of antisense RNA to C/EBPα did not affect the levels of C/EBPβ mRNA. The results of Lee, *et al.* (1997), in which C/EBPα was conditionally knocked-out in adult mice *via* the Cre/loxP recombination system, also indicated no change in C/EBPβ mRNA levels as a result of the down regulation of the α-isoform of C/EBP. These observations are not in agreement with our observations in rat hepatoma H4IIE cells where we observed a doubling of both C/EBPβ mRNA and protein as a consequence of antisense C/EBPα RNA expression (Figure 4.9). The fact that we were able to detect an increase in C/EBPβ mRNA levels in our C/EBPα antisense RNA expressing cell line may merely be a consequence of our use of the more sensitive ribonuclease protection analysis technique for determining changes in mRNA accumulation. As already presented, the changes in C/EBPβ mRNA were parallelled by an increase in the levels of C/EBPβ protein as well. Our observation of an increase in C/EBPβ expression along with down-regulation of C/EBPα may help lend support to the

observations of Wang, *et al.* (1995), who reported an interesting discrepancy. These researchers observed an almost total inhibition of PEPCK-C mRNA in the livers of newborn C/EBPα neonatal knockout mice, but after 7 hours post-partum the levels of PEPCK-C climbed back to control values. It was the opinion of these workers that other members of the C/EBP family such as C/EBPβ, might be capable of substituting for C/EBPα at these later time points so as to reestablish the levels of PEPCK-C expression. This would certainly be a possibility if a molecular mechanism exists to cause the levels of C/EBPβ expression to undergo a compensatory rise as a result of the down-regulation of C/EBPα. Currently no clear information exists concerning the potential regulation of the C/EBPβ gene promoter by other C/EBP isoforms, but given the data presented in this thesis work, which has been discussed in this section and in section 5.2.1.1, it is proposed that this might occur.

5.2.1.3 The Effects of Stable C/EBPβ Antisense RNA on C/EBP Isoform Expression

The stable expression of antisense RNA specific for the β-isoform of C/EBP also had significant effects on the expression of the two main hepatic C/EBP isoforms (Figure 4.10). The expression of C/EBPβ antisense RNA in the βA C4 H4IIE cell line produced significant decreases in both C/EBPβ mRNA and protein levels. Whether the decrease in C/EBPβ mRNA levels in the βA C4 cell line relative to controls is due to increased susceptibility of C/EBPβ transcripts to nuclease digestion, brought about by formation of

duplex with its antisense RNA, or is due to a potential lack of auto-regulation of the C/EBPβ gene by its own protein as seems to occur in the case of the C/EBPα gene, is uncertain. As already stated, no clear evidence exists regarding the regulation of the C/EBPβ gene by C/EBP isoforms, although such a mechanism has been at least suggested in the literature and the results of this thesis work would certainly lend support to this hypothesis. A possible avenue of future investigation into potential auto-regulatory properties of the C/EBPβ gene would involve measuring the basal transcription rate of this gene in the βA C4 H4IIE cell line in comparison to that in wild type H4IIE cells. The levels of C/EBPα protein are significantly increased in the βA C4 cell line relative to wild type cells. This inductive effect on the "opposing" C/EBP isoform in a cell line expressing antisense RNA to a given isoform is similar to that observed in the αA B1 cell. This effect might again be some sort of compensatory mechanism which serves either to maintain or balance the levels of the two main hepatic C/EBP isoforms in the liver cell. However, unlike the effect on C/EBPβ expression observed in the αA B1 cell which appears to be at least primarily at the level of its mRNA, the effects upon C/EBPα expression in the βA C4 cell appeared to be due to a post-transcriptional up-regulation of C/EBPα protein levels. No change in the levels of C/EBPα mRNA in the βA C4 cell line relative to wild type H4IIE cells were ever observed, however a consistent up-regulation of its protein did occur. Post-transcriptional regulation of C/EBP isoform expression by another C/EBP protein has recently been reported in two articles arising from related research groups. The work of Burgess-Beusse, *et al.* (1999), reported that the induction of C/EBPα expression in

cultured liver cells leads to an increase in LIP, an alternate translation form of C/EBPβ (section 2.3.1.3). The work of Welm, *et al.* (1999), has further expanded upon this observation and reported that C/EBPα-induced production of the LIP form of C/EBPβ occurred as a result of a post-translational proteolytic cleavage of full length C/EBPβ. Although not directly correlated with our own observations, the work of Burgess-Beusse, *et al.* (1999), and Welm, *et al.* (1999), do complement our findings if it is hypothesized that some form of reciprocal post-transcriptional regulation occurs to control the levels of C/EBPα and C/EBPβ protein in the liver. A rise in C/EBPα protein levels appears to induce a decrease in the more transcriptionally active form of C/EBPβ, and based on our own findings it seems that an induced decrease in C/EBPβ protein levels can bring about an up-regulation in the full length form of C/EBPα. It would be an interesting future prospect to more closely examine the ratios of C/EBP isoform alternate translation products within antisense expressing H4IIE cell lines to attempt to uncover more information regarding a mechanism by which an alteration in the expression of one C/EBP isoform might bring about reciprocal post-transcriptional changes in the expression and transcriptional activity of the other.

5.2.2 The Role of C/EBP Isoforms in the Mediation of the cAMP Responsiveness of PEPCK-C

The results presented in Figure 4.12 have clearly defined C/EBPα as the isoform required for the cAMP responsiveness of the endogenous PEPCK-C gene in rat hepatoma

H4IIE cells. This observation is supported by the lack of cAMP induction of PEPCK-C in both the GBF-F D4 and αA B1 cell lines and the robust cAMP response which can still occur in the βA C4 cell line. Further support of this hypothesis that C/EBPα is the regulator of PEPCK-C cAMP responsiveness is derived from the observation that the levels of C/EBPα protein were reduced in the GBF-F D4 cell line (Figure 4.8), in which PEPCK-C cAMP responsiveness was abolished, in addition to the functional inhibition upon C/EBP isoform transcriptional activity which exists due to GBF-F expression in these cells. Also in support of this hypothesis, the levels of C/EBPβ protein were significantly increased in the αA B1 cell line (Figure 4.9), and yet no response of PEPCK-C to cAMP was generated (Figure 4.12). Furthermore, the levels of C/EBPα protein were considerably elevated in the βA C4 cell line (Figure 4.10) in which a consistent and significant increase in the cAMP responsiveness of PEPCK-C was observed. There appears to be no global inhibition of cAMP signalling in either the αA B1 or GBF-F D4 cell lines as indicated by the cAMP induction of the C/EBPβ gene which was comparable to that seen in wild type H4IIE cells (Figure 4.13). As discussed in section 5.2.1.1, the possibility that the expression of GBF-F also inhibits fos/jun activity must be considered in the interpretation of the above data since the fos/jun heterodimer AP1 is an active factor in the PEPCK-C promoter's CRU, being required to mediate the synergy between CRE-1 and the LSR (section 2.2.3.1.3). However, given the effects of C/EBPα antisense RNA on PEPCK-C cAMP responsiveness, this possibility does not weigh greatly upon the interpretation of which C/EBP isoform is required to mediate the response. Thus the requirement for the

α-isoform of C/EBP in this hormonal response of the PEPCK-C promoter is strongly supported by the data presented in section 4.2 of this thesis.

Although not addressing the role of C/EBPα in the regulation of the PEPCK-C gene by cAMP, the work of Wang, *et al.* (1995) and Lee, *et al.* (1997) demonstrated the requirement of C/EBPα for the basal expression of PEPCK-C (supported by Figure 4.12). Interestingly, both studies have demonstrated a potential difference in the developmental requirement of C/EBPα for PEPCK-C basal expression. As mentioned in section 5.2.1.2, the expression of PEPCK-C is initially delayed in the neonatal knockout mice of Wang, *et al.* (1995), but appears several hours post-partum and slowly rises back to normal levels. This observation suggests that the role of C/EBPα in maintaining PEPCK-C basal expression levels can be eventually substituted for by another transcription factor several hours after birth, perhaps C/EBPβ as has been suggested in the literature and which may be supported by our own findings as discussed above. However, the results of Lee, *et al.* (1997), showed that PEPCK-C basal expression is still strongly inhibited in the adult conditional C/EBPα knockout animal, suggesting that any sort of functional compensation for the loss of C/EBPα in knockout animals is not sustainable. Interestingly, the levels of C/EBPβ mRNA were not elevated in either neonatal or adult C/EBPα knockout models, although the levels of C/EBPβ protein were not measured. These observations do not correlate with our observation of increased C/EBPβ mRNA and protein levels in stable H4IIE cells expressing C/EBPα antisense RNA (Figure 4.9), and may suggest important developmental regulatory mechanisms which exist *in vivo*.

As a final point of support for our observations concerning the absolute requirement of C/EBPα in PEPCK-C cAMP responsiveness, adult knockout of C/EBPβ has been shown to have no effect on the basal expression or cAMP inducibility of PEPCK-C (Liu, *et al.* 1999), which is in total agreement with our findings presented in Figure 4.12.

5.2.3 The Role of C/EBP Isoforms in the Mediation of the Glucocorticoid Responsiveness of PEPCK-C

The results presented in Figure 4.14 of this thesis suggest a general requirement of C/EBP isoforms in the mediation of the glucocorticoid responsiveness of the endogenous PEPCK-C gene in rat hepatoma H4IIE cells. While specific antisense RNAs to C/EBPα or C/EBPβ did not significantly affect the response of PEPCK-C to dexamethasone, expression of GBF-F reduced this response by approximately 50%. This suggests a general requirement for C/EBP isoforms in this response, although a specific C/EBP isoform may not be required based on the observations made in the antisense RNA expressing cell lines. These observations correlate well with the recently identified role of C/EBP isoforms as accessory factors for the PEPCK-C glucocorticoid response. Although it has been suggested that C/EBP isoforms may affect glucocorticoid responsiveness of PEPCK-C by acting through the AF2 *cis*-element (section 2.2.3.1.1), recent evidence suggests that this is likely not the case (Wang, *et al.* 1996). Rather it appears that C/EBP isoforms binding to the PEPCK CRE-1 may be involved as deletion of this region of the promoter can reduce glucocorticoid responsiveness by 50% (Imai, *et al.* 1993). Recent work by Yamada,

et al. (1999), based on synthetic promoter constructs and GAL4-fusion protein expression, suggested that C/EBPβ may be the specific accessory factor which aids in the mediation of the glucocorticoid response through the PEPCK-C CRE-1. Although the data presented herein suggests that either C/EBP isoform can participate, they generally support the hypothesized involvement of C/EBP isoforms in this transcriptional response of the PEPCK-C gene to glucocorticoids and does perhaps more accurately reflect endogenous regulatory mechanisms.

Finally, the data presented in Figure 4.14 showing the glucocorticoid responsiveness of PEPCK-C in stable H4IIE cell lines in comparison to wild type cells, taken in combination with results shown in Figures 4.12 and 4.13, showing the cAMP responsiveness of PEPCK-C in stable H4IIE cell lines in comparison to wild type cells and the cAMP responsiveness of the C/EBPβ gene in the αA B1 and GBF-F D4 cell lines, respectively, indirectly support the concept that the production of the H4IIE cell line models by stable transfection protocols has not affected global hormonal signalling.

5.3 Concluding Remarks

The results presented in this thesis provide a significant contribution to the understanding of the regulation of C/EBP isoforms in a liver background and have identified the C/EBP isoform required to mediate cAMP responsiveness of the endogenous PEPCK-C gene in liver. Furthermore, genetically modified rat hepatoma cells have been

produced which can be utilized to assess the requirements of C/EBP isoforms in various endogenous molecular mechanisms present within the liver cell.

This thesis work has demonstrated the tissue-specific nature of the regulation of C/EBPα by hormones which correlates well with its role as a *trans*-activator of many metabolically important genes whose expression patterns are regulated by many of the same hormones, and in many cases which must be uniquely tailored to the metabolic profile of a given tissue. This work has also demonstrated an unique alteration in the *trans*-activational activity of C/EBPα in the livers of streptozotocin-diabetic rats, which may prove useful in accounting for changes in the expression of genes regulated by C/EBPα which occur in the diabetic state.

The production of stable rat hepatoma H4IIE cell lines expressing molecules designed to inhibit the activity of C/EBP isoforms has provided for the assessment of the requirement of these transcription factors in various cellular processes. This thesis work has also demonstrated the need to build upon synthetically-based *in cyto* studies with experiments which can perhaps more accurately characterize endogenous molecular mechanisms, given the fact that experiments based on more artificial systems can only provide initial information regarding the potential for a given molecular mechanism to occur. In combination with *in vivo* based studies, cell culture based experiments directed towards endogenous genes can serve well to elucidate the complexity of regulational mechanisms within the intact cell.

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